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The Mechanism of Action of Methotrexate-induced BeWo Cell Differentiation

by

Neal S. Burres

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Abstract

During exposure to methotrexate (MTX), cultured human choriocarcinoma (BeWo) cells undergo a cytodifferentiative response that is a "caricature" of development of *in utero* quiescent syncytiotrophoblast. This response includes morphological changes, loss of proliferative capacity, and increased chorionic gonadotropin secretion and alkaline phosphatase activity. The objectives of this study were to establish the relationship between differentiation of BeWo cells and methotrexate toxicity and to determine the mechanism of methotrexate-induced differentiation. Inhibition of proliferation and expression of markers of differentiation during 48-hr exposures to drug displayed similar concentration-effect relationships with maximal changes seen at 1 μM. Drug exposures that completely inhibited proliferation also inhibited thymidylate synthetase, decreased incorporation of [^{14}C]formate into DNA, RNA, and protein by greater than 90%, and blocked progression through S-phase of the cell cycle. Protection experiments with leucovorin, hypoxanthine, and thymidine indicated that the differentiative response was due exclusively to thymidylate deprivation, whereas either purine or thymidylate starvation inhibited proliferation. Purine starvation antagonized MTX-induced formation of giant cells, but did not alter expression of alkaline phosphatase or morphological differentiation. Increases in the population density of cultures also antagonized MTX-induced expression of syncytiotrophoblastic markers, a phenomenon that was related to both depletion of hypoxanthine from culture fluids and density-dependent effects. Although BeWo cells were sensitive to the biochemical and antiproliferative effects of MTX, they were resistant to its cytotoxic effects since colony-forming ability was unaffected by 48-hr exposures to MTX (10^{-12} - 10^{-5} M). Colonies arising from drug-treated cultures were composed of cells morphologically indistinguishable from untreated BeWo stem cells, indicating that, following removal of drug, syncytiotrophoblast-like cells reverted to the proliferative stem-cell phenotype. Thus, MTX-induced differentiation of BeWo cells coupled with reversion to the stem-cell phenotype represents a novel "biological" mechanism of resistance to MTX.

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Abbreviations

A	Area
Ade	Adenine
AICAR	Phosphoribosyl-aminoimidazole carboxamide
ATP	Adenosine triphosphate
cAMP	3',5'-Cyclic adenylic acid
cpm	Counts per minute
CTL	Cytotrophoblast-like
CTP	Cytidine triphosphate
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dFBS	Dialyzed fetal bovine serum
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
DNA	Deoxyribonucleic acid
dThd	Deoxythymidine
dUrd	Deoxyuridine
EDTA	Disodium ethylenediamine tetraacetate
f	Form factor
FAICAR	Phosphoribosyl-formamido-imidazole carboxamide
FGAR	Phosphoribosyl-formylglycineamide
FUdR	Fluorodeoxyuridine
GAR	Phosphoribosyl-glycineamide
GTP	Guanosine triphosphate
Hepes	N-2-Hydroxyethyl piperazine-N'-2-ethane sulfonic acid

Hx	Hypoxanthine
i	Current
L	Length
mA	Milliamperes
MTX	Methotrexate
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide diphosphate
NRH	Novikoff Rat Hepatoma
P	Volume fraction of particles suspended in an electrolyte
p	Probability
pl	picoliter
R	Resistance
r	Specific resistivity
r^2	Coefficient of determination (least squares linear regression)
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
S.D.	Standard deviation
S.E.	Standard error
STL	Syncytiotrophoblast-like
Tris	Tris(hydroxymethylaminoethane hydrochloride)
ΔU	Coulter pulse height (volts)
UTP	Uridine triphosphate
V	Volume

I. Methotrexate and Induction of Differentiation

A. Introduction

The biochemical effects of methotrexate, one of the first clinically useful antineoplastic drugs, are well understood (1-3). Methotrexate inhibits dihydrofolate reductase [E. C. 1.5.1.3], blocking synthesis of tetrahydrofolate from folate or dihydrofolate. Tetrahydrofolate cofactors are required for one-carbon transfers during the synthesis of thymidylate, purines, serine, glycine, and methionine (4). In mammalian cells, folate cofactors remain at the tetrahydrofolate reduction level in all reactions except the synthesis of thymidylate. During the enzymatic methylation of deoxyuridylate, the tetrahydrofolate cofactor provides the one-carbon unit and the reducing equivalent required for this reaction (5). Since tetrahydrofolate cofactors are interconvertible (with the exception that formation of N^5-methyltetrahydrofolate is irreversible) and are present in cells at catalytic concentrations, continued synthesis of thymidylate in the presence of methotrexate leads to depletion of tetrahydrofolate cofactors resulting in disruption of folate-dependent one-carbon metabolism. The antiproliferative and cytotoxic effects of methotrexate are attributed to thymidylate and purine starvation (6-19).

In addition to its toxicity against neoplastic cells, methotrexate has also been shown to induce cytodifferentiative changes in several types of cultured cells (20-22). The cytodifferentiative mechanism(s) of action of methotrexate has not been elucidated. There is now a large literature concerned with the induction of differentiation of cultured cells by treatment with a broad array of chemicals (20-37), including a number of chemotherapeutic drugs (20-22, 31-37). In chemotherapy, the use of differentiation inducers has been proposed as a means of controlling neoplasia by stimulating terminal differentiation of tumor stem cells (38). Since this concept was first proposed (39-44), evidence in support of differentiation induction as an approach to the control of certain types of neoplastic diseases has been obtained in several *in vitro* and *in vivo* experimental systems (45-60). Knowledge of the molecular processes underlying normal and abnormal differentiation could have profound consequences with regard to the development of new protocols for the treatment of cancer. This prospect, though exciting, is distant. However, an understanding of the biochemical mechanism(s) of action of drugs that induce cytodifferentiative changes in cultured cells may provide a rationale for adjunctive chemotherapy based on controlling the proliferative state of neoplastic stem cells.

This investigation is concerned with the mechanism of methotrexate-induced differentiation and the relationship between differentiation and methotrexate toxicity in the BeWo cell line. The BeWo cell line was derived from a brain metastasis of a gestational choriocarcinoma of a patient who had become clinically resistant to methotrexate (61, 62). Choriocarcinoma, a tumor arising from the placenta, retains many biological and biochemical characteristics associated with *in utero* trophoblast (63). Placental trophoblast and choriocarcinoma are comprised of 2 cell types: the cytotrophoblast, a comparatively undifferentiated stem cell, and the mature syncytiotrophoblast, which is nonproliferative (63, 64). Proliferating BeWo cultures also contain two cell types. The predominant phenotype, 96-99% of the total population, is morphologically similar to *in utero* cytotrophoblast (22, 62). Cytotrophoblast-like (CTL) cells are proliferative, mononucleate, and express relatively low levels of chorionic gonadotropin and placental alkaline phosphatase (22, 65). The remaining 1-4% of cells resemble the syncytiotrophoblast and are nonproliferative, large, and multinucleated (22). The appearance of these syncytiotrophoblast-like (STL) cells in serially transplanted BeWo cultures indicates that proliferating CTL cells possess the ability to spontaneously differentiate. Exposure of BeWo cultures to methotrexate inhibits proliferation and induces cells to convert to the STL phenotype at a high frequency (22). STL cells present in methotrexate-treated cultures, maximally 80-90% of the total population, are morphologically indistinguishable from spontaneously occuring STL cells present in untreated cultures (22). In addition to morphological differentiation, BeWo cells exposed to methotrexate secrete increased amounts of chorionic gonadotropin and express elevated alkaline phosphatase activity, markers of mature placental function associated with the syncytiotrophoblast (65). Although the biological effects of methotrexate have been described, the underlying biochemical cause(s) of methotrexate-induced BeWo cytodifferentiation has not been previously studied in detail.

B. Objectives

Knowledge of the mechanisms of action of anticancer drugs has provided a rationale for improving the usefulness of existing drugs and has led to the development of structural analogs with increased therapeutic indices. The goal of current chemotherapeutic regimens is total eradication of neoplastic cells through the use of cytotoxic agents. An alternative approach is control of tumor growth by inducing terminal differentiation of neoplastic stem cells. Two related questions are central to the latter proposal: *(a) Are the cytodifferentiative mechanisms of action of cancer chemotherapeutic agents different from their cytotoxic mechanisms? and; (b) Can induction of terminal differentiation be separated from cell death?* If the cytodifferentiative mechanisms of action of drugs are different from "established" cytotoxic mechanisms of action, then a new basis for the development of treatment protocols and screening for active structural analogs is possible. Also, if induction of terminal differentiation is through nontoxic mechanisms, then drug regimens could be developed that optimize induction of differentiation rather than destruction of tumor tissue. This latter possibility may be of importance in the development of alternative therapies for patients for whom aggressive cytotoxic chemotherapy is contraindicated. Thus, an understanding of the mechanism(s) of action of cytodifferentiative agents and the relationship between differentiation and toxicity may provide a basis for a new approach to cancer chemotherapy based on cell maturation rather than cell kill. The primary objectives of this investigation were as follows: *(a) to develop quantitative markers for the BeWo CTL-to-STL differentiative response, (b) to determine the underlying biochemical cause(s) of methotrexate-induced* *expression of such markers, and (c) to determine if the cytodifferentiative response induced in BeWo cells by methotrexate could be separated from methotrexate cytotoxicity.*

While investigating the mechanism of action of methotrexate-stimulated expression of syncytiotrophoblastic markers, it became apparent that culture conditions profoundly affected the response of BeWo cells to the drug. Most notably, dialysis of serum used to supplement the basal growth medium and an increase in the number of cells per culture reduced expression of syncytiotrophoblastic markers during exposure to methotrexate. Hence, experiments were undertaken to: *(a) identify the serum factor(s) lost during dialysis, and (b) determine the basis of the inhibition of the CTL-to-STL differentiative response observed in crowded cultures.*

C. Experimental Approach

The BeWo cell line was derived from neoplastic tissue obtained from a patient who had become refractory to methotrexate chemotherapy (61, 62), and BeWo cells have been described as "methotrexate-resistant" in the literature (66). However, the degree of resistance of cultured BeWo cells to methotrexate has not been characterized and the biochemical mechanism(s) of action of methotrexate in these cells has not been described. A novel mechanism of action has been suggested (22). Thus, the effects of methotrexate on one-carbon metabolism of BeWo cells were investigated. Exposure to a growth-inhibitory concentration of methotrexate inhibited the apparent intracellular activity of thymidylate synthetase [E. C. 2.1.1.45], inhibited flow of [^{14}C]formate through folate-dependent biosynthetic pathways, inhibited RNA and protein synthesis, and blocked progression through S phase of the cell cycle. These observations were consistent with inhibition of dihydrofolate reducatase by methotrexate. Protection experiments with hypoxanthine and thymidine indicated that methotrexate inhibition of proliferation was due to depletion of cellular pools of thymidylate and purines. Thus, cultured BeWo cells were as sensitive to the biochemical and antiproliferative effects of methotrexate as most other cell types.

Before determining if inhibition of dihydrofolate reductase was the underlying cause of the CTL-to-STL differentiative response, markers associated with the syncytiotrophoblast of *in utero* placenta were characterized. The markers chosen were formation of giant cells, acquisition of the STL phenotype, and expression of placental alkaline phosphatase. Under culture conditions permissive for the CTL-to-STL differentiative response, these markers displayed similar time-courses of appearance following exposure to methotrexate. Furthermore, inhibition of proliferation and the two markers studied (increased cell volume and acquisition of the STL phenotype) displayed similar concentration-effect relationships. These observations suggest that methotrexate action at a single biochemical target resulted in expression of cytodifferentiative markers and inhibition of proliferation.

To determine if inhibition of dihydrofolate reductase by methotrexate was the underlying cause of the CTL-to-STL differentiative response, the ability of hypoxanthine and thymidine or of leucovorin to protect against the cytodifferentiative effects of methotrexate were assessed. Methotrexate disrupted both thymidylate and purine synthesis, but thymidylate starvation was established as the biochemical effect responsible for induction of the cytodifferentiative response of BeWo cells. Upon removal of methotrexate, after exposures to concentrations that are lethal to sensitive cell types, STL cells reverted to the CTL phenotype. Although thymidylate and purine starvation are toxic in other cell types, BeWo cells were not killed as determined in a colony-forming assay. Instead, methotrexate induced phenotypic changes that were a caricature of normal syncytiotrophoblastic development, suggesting that reversible differentiation to the STL phenotype may represent a novel biological form of resistance to the cytotoxic effects of methotrexate.

During this investigation, it was observed that expression of syncytiotrophoblastic markers by BeWo cells were profoundly altered by culture conditions. Methotrexate-induced formation of giant cells, but not expression of alkaline phosphatase or morphological differentiation to the STL phenotype, was dependent on a dialyzable serum factor. In addition, the CTL-to-STL differentiative response induced by methotrexate was less apparent in crowded cultures.

Since formation of giant cells was associated with increases in cellular RNA content and purines are known to be present in undialyzed fetal bovine serum, experiments were undertaken to determine if a purine was the factor lost during diaysis. Specific methods of removing purines and reconstitution of dialyzed serum with purines and purine nucleosides indicated that hypoxathine was the dialyzable factor required for formation of giant cells. Determination of the purine content of fetal bovine serum by reversed-phase high performance liquid chromatography indicated that the amount of hypoxanthine present in serum was nearly sufficient to account for methotrexate-induced increases in cell volume.

To separate the effects of cell population and cell density on the CTL-to-STL differentiative response, a culture system was developed that allowed manipulation of cell density in BeWo cultures with constant cell numbers and volumes of growth medium. Using this method, it was determined that the CTL-to-STL differentiative response was inhibited in a density-dependent manner. The effects of cell density on uptake of methotrexate and utilization of purine bases from the growth medium suggested that reduced availability of the inducer and exogenous factors required for formation of giant cells may have been important in density-dependent inhibition of the BeWo cytodifferentiative response. However, the large number of effects of methotrexate on the BeWo cell surface and cell-substratum interactions (67) suggests that culture microenvironment may have more profound effects on the CTL-to-STL differentiative response.

D. References

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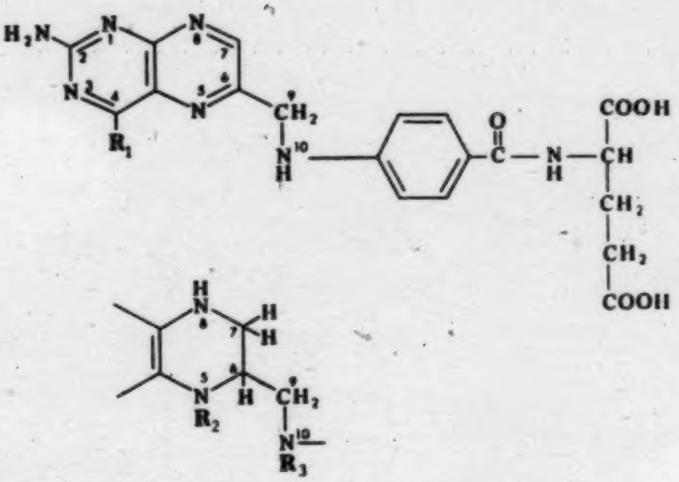
II. Mechanism of Action of Methotrexate

A. Introduction

The mechanism of action of methotrexate, one of the first clinically useful anticancer drugs, has been studied extensively (1-5). Methotrexate is an analog of folic acid (Figure 1), and its cytostatic and cytotoxic effects are attributed to inhibition of dihydrofolate reductase, resulting in depletion of thymidylate and purines and inhibition of DNA, RNA, and protein synthesis. Methotrexate cytotoxicity requires cellular uptake of methotrexate, inhibition of dihydrofolate reductase, and depletion of tetrahydrofolate cofactors such that thymidylate and/or purine synthesis are inhibited (Figure 2). In addition, cells must enter or be in S phase of the cell cycle during the period of exposure to methotrexate. In cultured cells, resistance to the toxic effects of methotrexate has been demonstrated to occur by mechanisms involving each of the obligatory steps required for cell kill (6-10). Although there is little doubt that disruption of thymidylate and purine synthesis is toxic, the pharmacology of methotrexate is complex and the exact mechanism(s) of cell death is unknown (11-12).

Depletion of cellular tetrahydrofolates also disrupts certain pathways of amino acid synthesis. Enzymatic methylation of homocysteine, the only *de novo* source of methionine, interconversion of serine and glycine, and formation of glycine from carbon dioxide and ammonia all require reduced folate cofactors (13-15). Since alternate pathways of serine and glycine biosynthesis exist (13) and methionine, as well as other amino acids, are present in the diet and are available in media used for cell culture, disruption of amino acid synthesis is not generally considered important for drug toxicity *in vivo* or *in vitro*. In isolated enzyme systems, methotrexate directly inhibits thymidylate synthetase, phosphoribosyl-aminoimidazole carboxamide [E. C. 2.1.2.3], and phosphoribosyl-glycineamide [E. C. 2.1.2.2] formyltransferase, methylene tetrahydrofolate reductase [E. C. 1.1.1.68], and folate interconverting enzymes (16-20). In intact cells, secondary effects of methotrexate include inhibition of


H₂N
N
1
2
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H
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8
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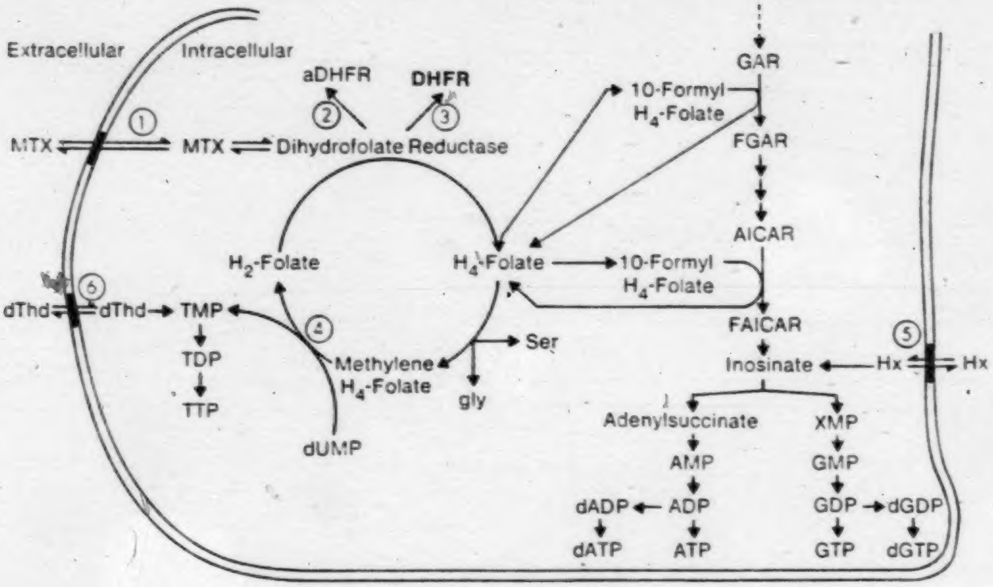
H
H
6
H
5
N
R₂
9
CH₂
N
10
R₃

Name	R_1	R_2	R_3	Unsaturation
Folate	OH		H	$\Delta^{5,6}$, $\Delta^{7,8}$
Dihydrofolate	OH		H	$\Delta^{5,6}$
Tetrahydrofolate	OH	H	H	
^{5}N-Methyl-tetrahydrofolate	OH	CH_3	H	
^{5}N-Formyl-tetrahydrofolate	OH	CHO	H	
^{10}N-Formyl-tetrahydrofolate	OH	H	CHO	
^{5}N,^{10}N-Methylene-tetrahydrofolate	OH	$—CH_2—$		
^{5}N,^{10}N-Methenyl-tetrahydrofolate	OH	—CH		
Methotrexate	NH_2		CH_3	$\Delta^{5,6}$, $\Delta^{7,8}$
Aminopterin	NH_2		H	$\Delta^{5,6}$, $\Delta^{7,8}$

Figure 1. Structure of folates, folate coenzymes, and antifolates.

glycolysis and uptake of glucose (21, 22), increased intracellular concentrations of 5-phosphoribosyl 1-pyrophosphate (23), deoxyuridylate (24-28), dUTP (29), misincorporation of uracil into DNA (28), increased ribonucleotide reductase [E. C. 1.17.4.1] activity (30), and inhibition of methionine uptake (31). The importance of these effects for drug toxicity and selectivity is unknown. The purpose of this review is twofold: to summarize the mechanism of action(s) of methotrexate, and to assess attempts to correlate the biochemical effects of the drug with cell kill.



Extracellular
Intracellular
aDHFR
DHFR
①
②
③
MTX
MTX
Dihydrofolate Reductase
H2-Folate
H4-Folate
10-Formyl H4-Folate
10-Formyl H4-Folate
GAR
FGAR
AICAR
FAICAR
⑥
dThd
dThd
TMP
TDP
TTP
④
Methylene H4-Folate
dUMP
Ser
gly
⑤
Inosinate
Hx
Hx
Adenylsuccinate
XMP
AMP
GMP
dADP
ADP
GDP
dGDP
dATP
ATP
GTP
dGTP

Figure 2. Schematic representation of the mechanisms of action and resistance to methotrexate. MTX inhibits dihydrofolate reductase, blocking synthesis of H_4-Folate from H_2-Folate or folate. Synthesis of thymidylate from deoxyuridylate results in oxidation of methylene H_4-Folate and, in the presence of MTX, leads to depletion of H_4-Folate cofactors. 10-Formyl H_4-Folate is required for one-carbon transfers during the synthesis of FGAR and FAICAR. Mechanisms of resistance to methotrexate are: (1) decreased uptake of drug, (2) altered binding of MTX to dihydrofolate reductase, (3) elevated levels of dihydrofolate reductase, and (4) decreases in the ratio of thymidylate synthetase to dihydrofolate reductase activity. In addition methotrexate toxicity can be circumvented by (5) salvage of Hx and (6) salvage of dThd. ***Abbreviations:*** MTX;methotrexate, aDHFR;altered dihydrofolate reductase, **DHFR**; elevated dihydrofolate reductase, H_4-Folate;tetrahydrofolate, GAR; phosphoribosyl-glycineamide, FGAR;phosphoribosyl-formylglycineamide, AICAR; phosphoribosyl-aminoimidazole carboxamide, FAICAR;phosphoribosyl-formamido-imidazole carboxamide.

B. Interaction of Methotrexate with Dihydrofolate Reductase

1. Isolated enzyme systems

Before the central role of dihydrofolate reductase in folate metabolism was elucidated, methotrexate was shown to block activation of folic acid to "citrovorum factor," (^{5}N-formyltetrahydrofolate) (32). After identification of dihydrofolate reductase, aminopterin and MTX were shown to be potent inhibitors of the enzyme (33).[1] Early studies suggested that methotrexate was a noncompetitive inhibitor of dihydrofolate reductase (36-38), but subsequent work demonstrated that methotrexate competitively interacted with dihydrofolate, and that apparent noncompetitive kinetics were a result of extremely tight-binding of the drug to the enzyme (39, 40). At relatively low pH (5.3-6.2), methotrexate binds to dihydrofolate reductase stoichometrically, whereas at pH 6.7 to 8, methotrexate is a competitive inhibitor with respect to folate or dihydrofolate (39). Values for the Ki of inhibition of dihydrofolate reductase from a variety of sources by methotrexate are in the nM to pM range (41). Although binding of methotrexate to dihydrofolate reductase is extremely tight, it is reversible as evidenced by dissociation of the enzyme-inhibitor complex during DEAE-cellulose chromatograpy (42) and displacement of [^{3}H]methotrexate from purified enzyme by dihydrofolate (43, 44).

2. Cultured cells

The antiproliferative and cytotoxic effects of methotrexate are attributed to inhibition of dihydrofolate reductase. The affinity of purified dihydrofolate reductase for methotrexate is extremely high and it was expected that the amounts of cell-associated methotrexate required for toxicity would equal the intracellular concentration of the enzyme. However, the relationship between inhibition of DNA synthesis and cell-associated methotrexate is hyperbolic, and the amount of drug required for half-maximal inhibition of DNA synthesis is

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[1] Although folate antagonists can alter folate metabolism by inhibiting uptake of reduced folates, folate interconversions, and folate-dependent reactions, the clinically useful antifolates (anticancer drugs: methotrexate, aminopterin, dichloromethotrexate; antimalarial drugs: pyrimethamine and cycloguanil) appear to act primarily by inhibiting dihydrofolate reductase (34, 35).

10,000-fold greater than the Ki of methotrexate for inhibition of dihydrofolate reductase (44, 45). A similar dose-response for inhibition of [^{14}C]formate incorporation into DNA RNA, and protein was observed (46). Based on these and similar observations, it was concluded that interaction with either a "low-affinity" form of dihydrofolate reductase or a different target was required for methotrexate toxicity (47). Although the development of resistance to methotrexate, in certain instances, is associated with an altered form of dihydrofolate reductase that does not bind methotrexate tightly (48-50), in most cells there is no evidence for the presence of a "low-affinity" form of dihydrofolate reductase (51).

The competitive relationship between methotrexate and dihydrofolate for binding to dihydrofolate reductase suggests that it is unneccessary to invoke action at a low-affinity site to explain the requirement for cell-associated drug in excess of the dihydrofolate reductase level for toxicity (44). Dihydrofolate can displace methotrexate from purified dihydrofolate reductase (43, 44), and computer simulations based on the kinetic properties of dihydrofolate reductase and intracellular concentrations of folate cofactors predict that only 1-5% of the available enzyme activity is sufficient to meet cellular demands for tetrahydrofolate (44). Although methotrexate has a high affinity for purified dihydrofolate reductase and appears to stoichometrically bind to the enzyme, the drug apparently acts as a rapidly reversible, competitive inhibitor of dihydrofolate reductase in intact cells (44).

On the basis of direct inhibition of thymidylate synthetase in cell-free extracts, and the ability of thymidine to partially reverse methotrexate toxicity, thymidylate synthetase was suggested to be a primary site of action of methotrexate in L60T cells (47). However, 10 μM "free" (unbound to dihydrofolate reductase) intracellular concentrations of methotrexate would probably be necessary for significant inhibition of thymidylate synthesis (52). Since the extracellular concentration of methotrexate required to inhibit proliferation of mammalian cells, including L60T cells, is about 10 nM, and the affinity of methotrexate for dihydrofolate reductase is very high, it is unlikely that unbound intracellular concentrations of drug are sufficient to directly inhibit thymidylate synthetase (53).

C. Inhibition of Dihydrofolate Reductase by Methotrexate in Cultured Cells

Folate cofactors are present in tissues and cells at low concentrations (54), and many are chemically unstable (55). The equilibrium constant for production of tetrahydrofolate from dihydrofolate by purified dihydrofolate reductase at pH 7 is 5.6 x 10^4 (56), and dihydrofolate has not been detected in proliferating cells (57). The first direct evidence that dihydrofolate levels increased in methotrexate-treated cells was obtained by incubating L1210 cells with [^{3}H]^{5}N-methyltetrahydrofolate. Although [^{3}H]dihydrofolate could not be detected in untreated cells, about 20% of administered radioactivity is recovered from methotrexate-treated cells as dihydrofolate (57). Similarly, if untreated Ehrlich ascites cells are incubated with [^{3}H]dihydrofolate, the rate of conversion of dihydrofolate to tetrahydrofolate is so rapid that [^{3}H]dihydrofolate can only be found in cells when the amount of cell-associated methotrexate was greater than the concentration of dihydrofolate reductase (58). Recently, methotrexate has been demonstrated to deplete intracellular levels of tetrahydrofolate in Lewis lung carcinoma cells (59).

Direct measurements of intracellular nucleotide concentrations indicate that methotrexate has "antithymidylate" and "antipurine" effects. Almost invariably, methotrexate-treated cells have diminished levels of dTTP (24, 25, 60-69), whereas deoxyuridylate pools increase, probably because of reduced utilization in thymidylate synthesis (24-28). Although enzymes that phosphorylate deoxyuridylate to dUTP are present in proliferating cells, dUTP is undetectable presumably because dUTP phosphatase [E. C. 3.61.23] efficiently hydrolyses dUTP to deoxyuridylate and pyrophosphate (29). However, increases in dUTP content have been demonstrated in methotrexate-treated cells (29). Misincorporation of uracil into DNA of methotrexate-treated human lymphoblast cells has been reported (28), and it has been suggested that, in such cells, a futile cycle of excision-repair may result in degradation of DNA and cell death (29).

Although dGTP pools frequently decrease during exposure to methotrexate (24, 25, 60, 62-64, 66, 67) dATP pools have been reported, in some cases to decrease (24, 25, 60, 62, 63, 66), and in others, to increase (58, 61, 64, 66, 67). When rapidly proliferating cultures are treated with methotrexate, dATP concentrations either decrease or are unchanged, and when slowly proliferating cultures are treated, dATP concentrations tend to increase (51).

Concentrations of dCTP also change in methotrexate-treated cells, probably because of changes in dTTP. The enzymes dCTP deaminase [E. C. 3.5.4.13] and ribonucleotide reductase are allosterically regulated by dTTP, and changes in the concentration of dTTP can either stimulate or inhibit dCTP production, and both effects have been demonstrated (24, 25, 60, 62-67).

Although ribonucleotide pools have also been demonstrated to decrease following exposure to methotrexate (25, 62, 68-70), relative changes are not as great as that observed with deoxyribonucleotide pools.

Utilization of precursors for folate-requiring biosynthetic reactions has also been assessed to determine the effects of methotrexate in cultured cells. Incorporation of [6-^{3}H]deoxyuridine into DNA is inhibited (26, 70-73) whereas utilization of radioactively-labeled thymidine is often stimulated (26, 72). Increased incorporation of thymidine probably reflects decreases in the dTTP pool and less dilution of radioactivity, before purines also become limiting for DNA synthesis. Decreased incorporation of deoxyuridine into the DNA of methotrexate-treated cells does not directly correlate with decreases in the tetrahydrofolate pool. Although concentrations of tetrahydrofolate decreased in the presence of methotrexate, they declined more slowly than did incorporation of [^{3}H]deoxyuridine into DNA (59). Explanations to account for this apparent discrepancy were that deoxyuridine incorporation into DNA measures the thymidylate synthetase activity of S phase cells, whereas the tetrahydrofolate pool is averaged over the total cell population, and thymidylate synthetase is also subject to inhibition by dihydrofolate suggesting that inhibition of the enzyme may be greater than that predicted from depletion of tetrahydrofolate alone (59).

Glycine is incorporated into the purine ring supplying C_2, C_5, and N_7. Methotrexate inhibits incorporation of [^{14}C]glycine into purines, DNA, and RNA, indicating that the two folate-dependent reactions of *de novo* purine synthesis are inhibited (74, 75). [^{14}C]Formate enters the folate one-carbon pool by conversion to ^{10}N-formyltetrahydrofolate, which is then interconvertible to all other folate cofactors. Methotrexate inhibits incorporation of [^{14}C]formate into DNA, RNA, and protein indicating that *de novo* purine, thymidine, and methionine synthesis are inhibited by depletion of tetrahydrofolate (76). Although precursor studies indicate that disruption of folate-dependent one-carbon metabolism inhibits DNA, RNA, and protein synthesis, the toxic effects of methotrexate in CCRF-CEM cells correlates with inhibition of DNA synthesis (64).

D. Mechanisms of Cell Death

Modulation of methotrexate toxicity by metabolites has been assessed to determine the relative importance of thymidylate and purine starvation in cell death. Coadministration of reduced folates or a purine and thymidine block the toxic effects of methotrexate against cultured cells (51). Antagonism of the cytotoxic effects of methotrexate by leucovorin and end-products of folate-requiring biosynthetic pathways is generally considered to reflect substitution of preformed metabolites for use as substrates and replenishment of tetrahydrofolate pools, respectively. However, in addition to replenishing depleted folate pools, leucovorin competes with methotrexate for transport, displaces intracellular methotrexate through a "counterflow" mechanism, results in an increase of the dihydrofolate pool, and may decrease the rate of methotrexate polyglutamation (51). Thus, protection against the toxic effects of methotrexate by leucovorin or other reduced folates may result from effects unrelated to simple substrate utilization.

Thymidine alone reduces methotrexate toxicity in some cells, and the mechanism for partial protection may be related to rates of thymidylate synthesis (24, 77, 78). Synthesis of thymidylate from deoxuridylate is the only folate interconversion that results in oxidation of tetrahydrofolate, and decreased synthesis of thymidylate would be expected to spare tetrahydrofolate requirements. It has been demonstrated that protection against the toxic effects of methotrexate by thymidine correlates with inhibition of thymidylate synthetase (24, 78).

Purines either potentiate (79, 80) or diminish (79, 81, 82) methotrexate toxicity in cultured cells. The differential effects of purines on cytotoxicity have been ascribed to intrinsic differences between cells, but it has been demonstrated that hypoxanthine either reduces or increases methotrexate toxicity in CCRF-CEM, PMC-22, and L1210 cells depending on the concentration of methotrexate (83).

The pharmacology of methotrexate is complex (Figure 3) and the exact mechanism(s) of methotrexate cytotoxicity is unknown. Inhibition of thymidylate (84), purine (85), and protein synthesis (86) and disruption of methylation reactions (87) are toxic, and methotrexate may inhibit all of these processes under certain conditions. Two proposed mechanisms of cytotoxicity are: (1) "thymineless death" which is augmented by exogenous purines and diminished by purine starvation, and (2) "purineless death" which is diminished by exogenous purines (12). In addition, it is well established that methotrexate is a proliferation-dependent cytotoxic agent; thus, the biochemical effects of the drug only result in toxicity if cells are proliferating during the period of exposure (88-90).

Thymineless death was first demonstrated with thymidine-requiring auxotrophs of *E. coli*. Thymine starvation was only toxic when cells synthesized RNA and protein suggesting that "unbalanced growth" was required for cell death (91). Subsequently, thymidylate starvation was demonstrated to result in "unbalanced growth" of cultured human tumor cells (92), and inhibition of RNA and protein synthesis antagonizes thymineless death in these cells (92). Increased toxicity in the presence of hypoxanthine correlated with increases in the concentration of dATP in CCRF-CEM cells suggesting that, in these cells, potentiation of methotrexate's toxic effects may be a synergism between deoxyadenosine and methotrexate toxicity (83). In addition to unbalanced growth, thymidylate starvation leads to chromosomal aberrations (93) and mitochondrial mutagenesis (94) in mammalian cells, both of which may contribute significantly to toxicity.

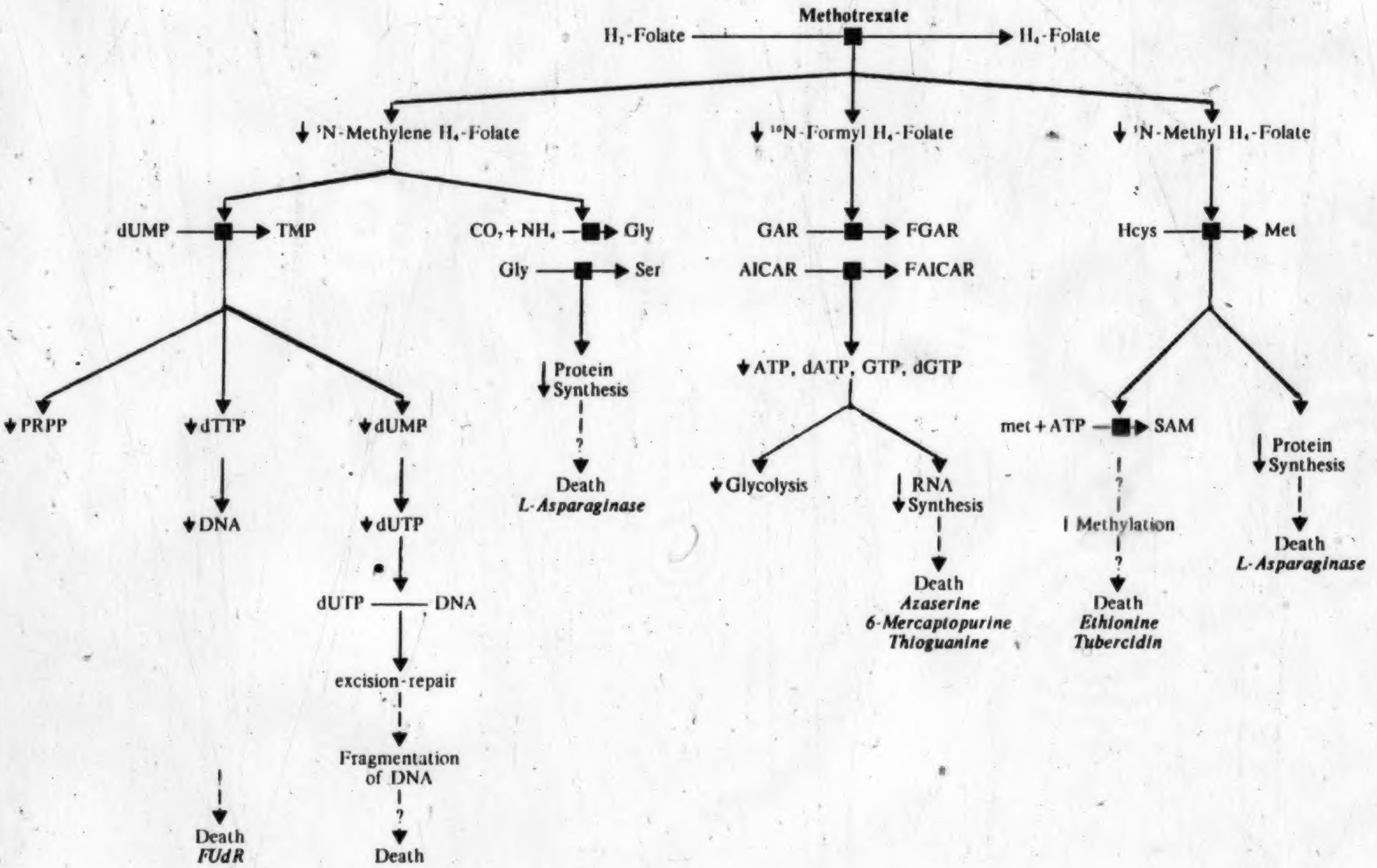
Methotrexate
H_2-Folate
H_4-Folate
↓ ^{5}N-Methylene H_4-Folate
↓ ^{10}N-Formyl H_4-Folate
↓ ^{5}N-Methyl H_4-Folate
dUMP
TMP
$CO_2 + NH_3$
Gly
Gly
Ser
GAR
FGAR
AICAR
FAICAR
Hcys
Met
↓ Protein Synthesis
↓ ATP, dATP, GTP, dGTP
↓ PRPP
↓ dTTP
↓ dUMP
met + ATP
SAM
↓ Protein Synthesis
?
Death
L-Asparaginase
↓ Glycolysis
↓ RNA Synthesis
↓ DNA
↓ dUTP
?
↓ Methylation
?
Death
L-Asparaginase
dUTP
DNA
Death
Azaserine
6-Mercaptopurine
Thioguanine
Death
Ethionine
Tubercidin
excision-repair
Fragmentation of DNA
?
Death
FUdR
Death

Figure 3. Cellular pharmacology of methotrexate.

Although methotrexate is often thought to kill cells because it disrupts thymidylate synthesis (95), the relative importance of inhibition of *de novo* purine synthesis for toxicity is still unknown. In some cell lines, exogenous purines reduce methotrexate toxicity (79, 81, 82) an observation which is incompatible with the concept of thymineless death. Thus, in these cell lines, the toxic effects of methotrexate have been ascribed to purine deprivation (12). In S-49 cells, toxic effects of purine starvation are associated with depletion of guanine nucleotides (96). The effects of an inhibitor of *de novo* purine synthesis (6-mecaptopurine ribonucleoside) and specific inhibitors of adenine (L-alanosine) and guanine (mycophenolic acid) nucleotide biosynthesis on inhibition of DNA synthesis and progression through the cell cycle were compared (96). Recovery of [^{3}H]thymidine incorporation into DNA of drug-treated cells depended on restoration of guanine nucleotide pools rather than adenine nucleotide pools, and the effects of mycophenolic acid and 6-mercaptopurine ribonucleoside on progression through the cell cycle were similar, whereas the effect of alanosine was strikingly different suggesting that the effects of purine starvation are primarily a result of guanine depletion (96).

E. Mechanisms of Resistance

The mechanisms of acquired resistance to methotrexate toxicity have been extensively studied and fall into three classes: (1) increased levels of dihydrofolate reductase, (2) alterations in the structure of dihydrofolate such that its affinity for methotrexate is decreased, and (3) reduced uptake of methotrexate (6-10). Less defined "intrinsic" mechanisms of resistance are associated with the relative insensitivity of non S-phase cells to methotrexate toxicity, the ratio of thymidylate synthetase to dihydrofolate reductase activity, and utilization of NADH as a substrate by dihydrofolate reductase rather than NADPH (6).

In 1961, increased levels of dihydrofolate reductase in L5178Y cells was first demonstrated to be a mechanism of resistance to methotrexate toxicity (97). Subsequently, cells selected for high levels of dihydrofolate were shown to have an amplification in gene frequency that is proportional to elevated levels of the enzyme and its messenger RNA (98).

Stable and unstable amplification of the dihydrofolate reductase gene has been demonstrated in a number of cell lines (98). In addition to increased levels of the enzyme, altered forms of dihydrofolate reductase with decreased affinity for methotrexate have been identified in methotrexate-resistant cultured cells (48-50). The decrease in affinity for methotrexate ranges from 4- to 100,000-fold (6).

Methotrexate enters cells through an energy-dependent carrier mediated transport system present for uptake of circulating reduced folates (99). Decreased Kt and Vmax values for transport of methotrexate have been observed with methotrexate-resistant cells, and in one instance, a complete loss of transport capacity was demonstrated (6, 99). Loss of the capacity to transport methotrexate can be tolerated because folate enters cells by an alternate transport system (6). Cells resistant to methotrexate because of decreased uptake of drug remain sensitive to "non-classical" lipophilic antifolates which passively diffuse into cells (6).

Acquired resistance to methotrexate involves decreased action at dihydrofolate reductase, the primary target of the drug, whereas "intrinsic" mechanisms of resistance to antifolates are less well characterized. Methotrexate is a proliferation-dependent cytotoxic agent that kills S-phase cells. It is well established that slowly proliferating or quiescent cells are resistant to the toxic effects of methotrexate (88-90). The degree of resistance can be considerable, for example, the viability of resting 3T6 cells was unaffected after 7-d exposures to 1 mM methotrexate, whereas greater than 90% of logarithmically proliferating 3T6 cells were killed after a 24-hr exposure to 1 μM methotrexate (90). Cells capable of rapidly synthesizing dihydrofolate reductase or cells with high ratios of dihydrofolate reductase to thymidylate synthetase activity are likely to be naturally resistant to methotrexate (6). As discussed previously, thymidylate synthesis is the only folate-dependent reaction that results in oxidation of tetrahydrofolate and decreased activity of this enzyme allows cells to tolerate greater inhibition of dihydrofolate reductase (24, 78). The affinity of dihydrofolate reductase for methotrexate is reduced in the absence of NADPH, and utilization of NADH may result in less sensitivity to inhibition of the enzyme by methotrexate (100).

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III. Model System: The BeWo Cell Line

A. Introduction

The BeWo cell line, derived from a malignant human choriocarcinoma, morphologically and biochemically resembles the trophoblast of normal placenta. In proliferating BeWo cultures, most cells are morphologically similar to *in utero* cytotrophoblastic cells (1-5). Also present, at a low frequency (< 5% of the total population), in proliferating cultures are cells that resemble *in utero* syncytiotrophoblast (5). Exposure of BeWo cultures to methotrexate inhibits proliferation and, within 48 hr, 80-90% of the cells are morphologically indistingushable from spontaneously arising STL cells (5). Morphological differentiation is accompanied with increased expression of biochemical markers of *in utero* syncytiotrophoblast (chorionic gonadotropin and heat-stable alkaline phosphatase) (6). Thus, the BeWo cell line represents an *in vitro* model system for investigating the differentiative effects of chemotherapeutic drugs and relationships between differentiation and cytotoxicity (5).

B. Normal Trophoblastic Development and Choriocarcinoma

Within 5 d of fertilization, the human embryo consists of approximately 120 cells in the form of a blastocyst (7). The outer cell layer of the blastocyst is the trophoblast, destined to become the placenta. Attached eccentrically on the inside of the blastocyst is the inner cell mass, destined to become the fetus (7). Upon implantation, the trophoblast invades the endometrium and differentiates into two layers (Figure 4). The outermost layer, or syncytiotrophoblast, is nonproliferative and multinucleated, whereas the inner layer, or cytotrophoblast, is composed of proliferative, moderately sized, mononucleated cells (8). Syncytiotrophoblast is apparently formed by fusion of cytotrophoblastic cells (8-14). As the placenta develops, villous structures (primary villi) consisting of an inner core of cytotrophoblastic cells and an outer layer of syncytiotrophoblast are formed (8).

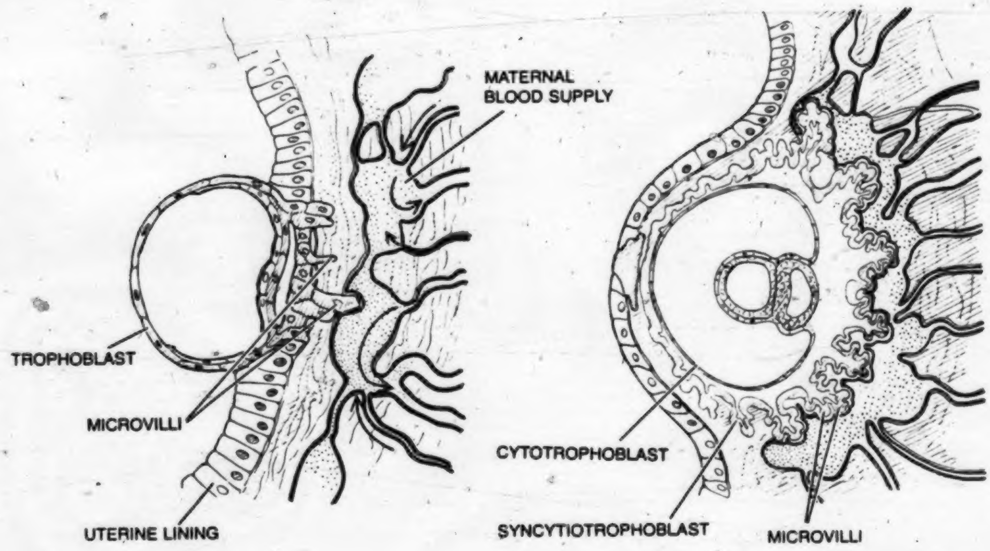
MATERNAL BLOOD SUPPLY
TROPHOBLAST
MICROVILLI
UTERINE LINING
CYTOTROPHOBLAST
SYNCYTIOTROPHOBLAST
MICROVILLI

Figure 4. Development of the human placenta. During implantation of the blastocyst in the endometrium, the trophoblast differentiates into two layers. The inner layer is composed of proliferative cytotrophoblastic cells. The outer layer, the syncytiotrophoblast, apparently arises by fusion of cytotrophoblastic cells (8-14). From The Placenta, P. Beaconsfield, G. Birdwood, R. Beaconsfield. Copyright© August 1980 by Scientific American, Inc. All rights reserved.

Morphological differentiation of the trophoblast is accompanied with functional specialization. Synthesis of chorionic gonadotropin (15-17), alkaline phosphatase (18-19), PP_5 (20), placental lactogen (21), and β_1 glycoprotein (22) are localized to the syncytiotrophoblast, but these studies have not demonstrated that cytotrophoblastic cells are completely unable to synthesize placental proteins (22).

Gestational choriocarcinoma, a malignant form of gestational trophoblastic disease, arises from placental trophoblast and is morphologically and biochemically similar to trophoblastic tissue (23). By definition, the tumor is composed of both cytotrophoblastic and syncytiotrophoblastic cells that are unable to form villi (23). Before the advent of chemotherapy, surgery and radiation were the primary forms of treatment, and death usually resulted within 1 yr after the patient presented with the disease (24). In 1961, methotrexate chemotherapy was reported to result in a complete and sustained remission in 47% of patients with metastatic choriocarcinoma (25). Today, the cure rate is approximately 90% (23, 26). Currently used chemotherapy regimens consist of daily intramuscular injections of 15 - 25 mg methotrexate for 5 d, and response is monitored by measuring chorionic gonadotropin (24). After 7 d, drug can be administered again, and the cycle is repeated until serum chorionic gonadotropin levels are normal (23). Although treatment of choriocarcinoma is usually successful, resistance to methotrextrate chemotherapy does occur, and patients resistant to chemotherapy have a median time of diagnosis to death of 6 months (26).

C. The BeWo Cell Line

The BeWo cell line was derived from a brain metastasis of a gestational choriocarcinoma of a patient who had become resistant to methotrexate chemotherapy (1, 27). The choriocarcinoma was originally maintained as a heterologous serial transplant (WO choriocarcinoma) in NIH-strain, female, golden hamsters (1).[2] During *in vitro* establishment of the continous cell line, a uniform cytotrophoblastic phenotype was selected by microsurgically excising and discarding non-trophoblastic areas of cultures (1). Proliferating BeWo cells are moderately sized, possess a single ovoid nucleus with prominent nucleoli, and are ultrastructurally similar to cytotrophoblastic cells of *in utero* placenta (1-5). A second cell type is also present in proliferating BeWo cultures at a low frequency (1 - 4% of the total population). These cells are apparently nonproliferative, larger than CTL cells, often multinucleated (4, 5) and ultrastructurally similar to the syncytiotrophoblast of *in utero* placenta (5). The presence of STL cells in serially-transplanted cultures indicates that BeWo CTL cells can spontaneously differentiate to the mature trophoblastic cell type.

Morphological and biochemical changes initiated by exposure to methotrexate indicate that differentiation of BeWo CTL cells to the STL phenotype can be chemically induced. Immediately following exposure to methotrexate, proliferation of BeWo cells is inhibited (5, 29), but cell growth and nuclear division are apparently unaffected resulting in the formation of giant cells that are often multinucleated (5). In cultures exposed to 0.1 or 1.0 μM methotrexate for 2 - 4 days, 80 - 90% of the cells are morphologically indistinguishable from spontaneously occuring STL cells present in proliferating BeWo cultures (5). Methotrexate also stimulates expression of chorionic gonadotropin and placental alkaline phosphatase, markers associated with the syncytiotrophoblast of *in utero* placenta (6). Thus, biochemical and morphological evidence indicate that methotrexate induces BeWo cells to undergo a cytodifferentiative response that parallels normal trophoblastic development (Table 1).

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[2] The WO-strain choriocarcinoma (27) secreted chorionic gonadotropin, placental lactogen, and steroid hormones. Excised tumors displayed broad sheets of cytotrophoblastic and syncytiotrophoblastic cells. In addition, a transitional cell with structural resemblence to both cell types was observed (28).

Table 1

Comparison of CTL and STL BeWo Cells with

***In Utero* Cytotrophoblastic and Syncytiotrophoblastic Cells**

	CTL BeWo Cells	STL BeWo Cells	*In Utero* Cytotrophoblast	*In Utero* Syncytiotrophoblast	References
Morphology[1]					
Cell Shape	Compact	Flattened	Compact	Irregular	1-5;5
Cell Size	Moderate	Giant	Moderate	Giant	1-5;5
Nuclear					
Shape	Ovoid	Multilobed	Ovoid	Multilobed	1-5;5
Number	Single	Multiple	Single	Multiple	1-5;5
Microvilli	Occasional	Abundant	?	Abundant	2,4,5;5
Ruffles	Abundant	Rare	?	?	5;5
Filopodia	Occasional	Common	?	?	5;5;
Lamellae	Occasional	Common	?	?	5;5
Rough Endoplasmic Reticulum					
Numbers	Moderate	Abundant	Moderate	Abundant	2;4;5
Morphology	Laminar	Dilated	Laminar	Dilated	5;5
Free Ribosomes	Abundant	Abundant	Abundant	Abudant	2,4,5;5
Golgi	Moderate	Abundant	Moderate	Abundant	2,4,5:5
Granules and Vesicles	Occasional	Moderate	Occasional	Moderate	5;5
Lipid Droplets	rare	common	rare	common	32,33;32, 33;8;8
Mitochondria					
Morphology	Oval	Elongated	Oval	Oval, small	5;5
	Round-Elongated				2,4
Numbers	Moderate	Abundant	Moderate	Abundant	5;5
Filament Network	Prominent	Limited	?	?	5;5
Desmosomes	Common	Rare	?	Common	2,4,5;5
Membrane Interdigation	Rare	Extensive	?	Extensive	5;5

Table 1 (cont'd.)

Comparison of CTL and STL BeWo Cells with

***In Utero* Cytotrophoblastic and Syncytiotrophoblastic Cells**

	CTL BeWo Cells	STL BeWo Cells	*In Utero* Cytotrophoblast	*In Utero* Syncytiotrophoblast	References
Hormone Expression					
Chorionic Gonodatropin	moderate	high	low	high	1,3,6,30;6; 16-18;16-18
Progesterone	+	?	+		32; ;34[2]
Estrone	+	?	+		33; ;35[2]
17β-Estradiol	+	?	+		33; ;35[2]
Enzyme Activity					
Heat-stable Alkaline Phosphatase	moderate	high	low	high	6,36,37;6,37; 18,19;18,19
Surface Marker					
Transferrin Receptor	high	high	?	high	38,†;†;39;39

†personal communication, Dr. R. M. Johnstone and M. Adam.

[1] ***Adapted from reference 5; the information about the ultrastructure of normal trophoblast was from references 13 and 31.***

[2] ***steroid synthesis has not been localized to a specific trophoblastic cell type.***

The mechanism of action of methotrexate during induction of the BeWo cytodifferentiative response has not been elucidated. It was suggested that increased expression of syncytiotrophoblastic markers is a direct result of inhibition of DNA synthesis (6), but others have found that DNA content of BeWo cells increased as differentiation to the STL phenotype occurred (5). An early investigation demonstrated that methotrexate-treated cells exhibited increased cellular protein:DNA ratios (29). Methotrexate inhibited utilization of deoxyuridine and stimulated utilization of thymidine, suggesting that thymidylate synthetase was inhibited during exposure to the drug (29). The biochemical effects of methotrexate on BeWo cells have not been further investigated.

A number of other agents stimulate synthesis and secretion of chorionic gonadotropin and the heat-stable form of placental alkaline phosphatase by BeWo cells. Although ectopic production of placental-type alkaline phosphatase and chorionic gonadotropin has been reported in a number of human tumor lines (40-45) and expression of either of these markers is considered a marker of neoplasia (46, 47), both placental-type alkaline phosphatase and chorionic gonadotropin are normal products of the trophoblast (8, 15-19, 48). Agents known to stimulate synthesis and secretion of chorionic gonadotropin by BeWo cells are dibutyryl cAMP (with or without coadministration of theophylline) (43, 49-50), sodium butyrate (49), actinomycin-D (51), and 1-β-D-arabinofuranosylcytosine (6); agents demonstrated to stimulate expression of heat-stable alkaline phosphatase are dibutyryl cAMP, 5-bromo-2'-deoxyuridine (36), and actinomycin-D (37).

Drug-induced expression of syncytiotrophoblastic markers is not unique to the BeWo cell line. Fluorodeoxyuridine, hydroxyurea, and 1-β-D-arabinofuranosylcytosine stimulate JAr cells, an independently derived choriocarcinoma cell line (52), to express elevated levels of chorionic gonadotropin (53). The effects of fluorodeoxyuridine and 1-β-D-arabinofuranosylcytosine are blocked by coadministration of thymidine and deoxycytidine, respectively, suggesting that inhibition of DNA synthesis is important for the response (52). Methotrexate has also been reported to induce morphological differentiation and stimulate expression of chorionic gonadotropin and heat-stable alkaline phosphatase in GCH-nu choriocarcinoma cells, another independently derived line (54).

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IV. Materials and Methods

A. Cells

1. Cultured cells

All cells were maintained at 37° in a humidified atmosphere of 5% CO_2 in air. Stock cultures of continous cell lines were grown in antibiotic-free growth medium and were reinitiated at 2 - 3 month intervals from frozen cells (cells suspended in growth medium supplemented with 10% dimethyl sulfoxide were stored in liquid nitrogen) that were free of *Mycoplasma* as determined by Dr. J. A. Robertson, Department of Medical Microbiology, University of Alb., Edmonton, Alberta, Canada. Unless otherwise indicated, cell culture materials were purchased from Grand Island Biological Co., Burlington, Ont. and Flow Laboratories, Inc., Mississauaga, Ont.. Gentamicin sulfate (50 μg/ml; Schering Corporation, Kenilworth, N.J.) was used in colony-forming assays, and, if indicated, penicillin (100 units/ml) and streptoymcin (400 μg/ml) were used for other experiments. All suspended cells were pelleted by centrifugation (120 x g, 10 min) in a Model UV International Centrifuge (International Equipment Co., Needham, Mass.). After monolayer cultures were disaggregated by trypsinization (0.05% trypsin, 0.02% EDTA in 0.15 M NaCl), 20 - 40 ml of warm growth medium was added to each suspension of cells before centrifugation. Unless otherwise indicated, growth medium was supplemented with 10% fetal bovine serum, if medium was supplemented with 10% dialyzed fetal bovine serum, then it was indicated by (dFBS).

a. BeWo Cells

Human choriocarcinoma BeWo cells, obtained from Dr. S. Friedman, Dept. of Pharmacology, University of Calgary, Alberta, were maintained as monolayers in plastic T-25 tissue culture flasks and were subcultured after dissociation with trypsin-EDTA at weekly intervals. Growth medium, unless otherwise indicated, was Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% fetal bovine serum. After an initial lag phase of 24 hr, the mean doubling time of proliferating cells was approximately 24 hr. Cultures that provided cells for experimental use were established by innoculating 1 x 10^6 cells per T-75 flask and were harvested 5-7 d later.

Experimental cultures (2 x 10^5 cells/culture) were initiated in 5 ml of growth medium in T-25 plastic flasks, 60 x 15 mm tissue culture dishes, or 2-oz glass prescription bottles (Brockway Glass, Brockway Penn.). For determination of the effects of methotrexate on proliferation and cell volume, 24-hr cultures were exposed to growth medium containing particular additives to be tested; in some experiments, fetal bovine serum was replaced with variously treated serum supplements. Culture fluids were replaced completely every 48 hr. Cell numbers were determined with an electronic particle counter (Model Zb or Model Zf, Coulter Electronics Inc, Hialeah, Fla.) after dissociation with trypsin-EDTA and suspension in 0.15 M NaCl.

Control and drug-treated cultures were observed daily by phase-contrast microscopy with a Leitz Diavert (Ernst Leitz (Canada) Ltd., Midland, Ont.) microscope using Kohler illumination. Cultures, after staining with Wright's stain, were photographed with a camera mounted on either a Leitz Orthoplan, Ernst Leitz (Canada) Ltd., or a Zeiss Universal (Carl Zeiss (Canada) Ltd, Don Mills, Ont.) microscope.

Serum supplements used for experiments were dialyzed, xanthine-oxidase [E. C. 1.2.3.2] treated, charcoal extracted, or untreated fetal bovine serum. Serum was exhaustively dialyzed against three 50-fold volumes of 0.15 M NaCl using a membrane tubing with a nominal molecular weight cutoff of approximately 3500 (Spectrapor 3, Spectrum Medical Industries Inc., Los Angeles, Calif.). Xanthine-oxidase treatment consisted of incubating 0.4 units (1 unit = 1.0 μmol of xanthine converted to uric acid/min at pH 7.5 at 25°) of enzyme per ml of serum at 20° for 30-60 min. Charcoal extraction was carried out by suspending 1 g of Norit-A (Fisher Scientific Co., Fairlawn, N. J.) in 10 ml of serum followed by filtration through a 0.22 μM filter (Millipore Corp., Bedford, Mass.).

b. HeLa Cells

Stock cultures of human cervical carcinoma HeLa S3 cells were maintained as described previously (1) as monolayers in Eagle's minimal essential medium supplemented with 10% calf serum with subculture at weekly intervals. Experimental cultures were established with 1 x 10^5 cells/T-75 flask and were harvested by trypsinization 5 - 7 d later. HeLa cell volumes were determined electrically and radiochemically as described for BeWo cells (section B).

For determination of the effects of methotrexate on viability, suspension cultures were initiated in spinner flasks, expanded into round-bottomed flasks and, in the latter, kept under continous agitation with vibrating mixers to prevent cell clumping (Vibro-mixer, Model E1; Chemapec, Hoboken, N.J.). In suspension cultures, cells proliferated with mean doubling times of approximately 24 hr. Suspension cells were established in monolayer cultures at 2 x 10^5 cells/T-25 flask in RPMI medium 1640 supplemented with 10% fetal bovine serum and adapted to growth for 2 - 3 d. Monolayer cultures were trypsinized to provide cells for the colony-forming assay. Viability of untreated and methotrexate-treated cells was assessed in a colony-forming assay exactly as described for BeWo cells (section A.4).

c. L1210 cells

Murine leukemia L1210/C2 cells were maintained in suspension cultures without shaking in Fischer's medium supplemented with 10% horse serum (2). Proliferating cells (doubling time, ca. 14 hr) were harvested by centrifugation and after resuspension in growth medium, were used for cell volume measurements as described below (section B).

d. Novikoff rat hepatoma cells

Wild-type Novikoff rat hepatoma (NRH) cells (N1S1-67), provided by Drs. P. G. W. Plagemann and R. M. Wolheuter in 1982, were adapted in the laboratory of Dr. A. R. P. Paterson to growth in Eagle's minimal essential medium supplemented with 5% horse serum, 5% calf serum, non-essential amino acids (0.1 mM) and glutamine (4 mM). The adapted line is referred to as Novikoff hepatoma UA. Cultures for determination of cell volume were expanded to 800 ml in growth medium with antibiotics in roller bottles rotated at 1.5 rpm. Cell concentrations were kept below 4 x 10^5 cells/ml.

e. S49 cells

Murine lymphoma S49 cells were maintained in suspension cultures without shaking in Fischer's medium supplemented with 10% horse serum (3). For experimental use, cultures were expanded to 800 ml in the same medium with antibiotics in roller bottles rotated at 1.5 rpm. Cell concentrations were kept below 8 x 10^5 cells per ml, and such cultures proliferated with a mean doubling time of approximately 18 hr. Cells were harvested by centrifugation and, after resuspension in growth medium, were used for cell volume measurements as described below (section B.).

f. HL-60 cells

Human promyelocytic leukemia HL-60 cells (4) were a gift from Dr. R. Gallo, NIH, Bethesda, MD. Growth medium was RPMI medium 1640 supplemented with 15% fetal bovine serum. Stock cultures were subcultured 3 or more times per week by diluting cells to 10^5/ml, and such cells proliferated with a mean doubling time of approximately 24 hr (5). The effects of methotrexate on viability of HL-60 cells were determined as follows (6). Drug exposures were initiated by combining suspensions of growing cells (2 x 10^5 cells/ml) with equal volumes of drug-free growth medium supplemented with 5% fetal bovine serum with or without 20 μM methotrexate. After 48-hr exposures, the colony-forming ability of HL-60 cells was determined as follows. Cells were diluted to densities of 40 and 80 cells/ml in growth medium supplemented with 30% conditioned growth medium (48-hr exposure to proliferating HL-60 cells), 30% fetal bovine serum, 200 μM hypoxanthine, and 20 μM thymidine. Cell suspensions were diluted again with an equal volume of RPMI medium 1640 containing 0.29% agar (Agar Noble, Difco Laboratories, Detroit, Mich), and 5 ml of the resulting cell suspension was added per 60 mm tissue culture dish (100 and 200 cells/dish). After cultures were incubated 14 d at 37°, macroscopic colonies were scored by examination with a dissecting microscope.

g. Morris hepatoma 3924A

Morris rat hepatoma 3924A cells (7), provided by Dr. G. A. Weber, were propagated as monolayers in McCoy's medium 5A supplemented with 10% fetal bovine serum with subculture at weekly intervals (8). The mean doubling times of such cultures were approximately 14 hr. Before viability of 3924A cells was assessed, cultures were initiated with 4.5 x 10^5 cells/T-75 flask, and after cells attached (2 hr), culture fluids were completely replaced with RPMI medium 1640 supplemented with 10% fetal bovine serum. Cells were propagated in RPMI medium 1640 supplemented with 10% fetal bovine serum to allow measurement of the effects of methotrexate on colony-forming ability under the exact conditions of BeWo cell experiments. After adapting cells for growth (3 d), experimental cultures were inoculated with 100 - 500 cells/60 mm plastic tissue culture dish, and the effect of methotrexate on colony forming ability was determined as described below (section A.4).

2. Blood cells

Peripheral blood, obtained from healthy volunteers by venipuncture, was collected into 10-ml heparinized (143 U.S.P. units per tube) Vacutainer tubes (Becton, Dickenson, Rutherford, N.J.). Whole blood was diluted in saline, and the volumes of erythrocytes were determined electronically and radiochemically (section B) without further treatment.

Lymphocytes were purified from whole blood which was obtained as described above. One ml of phosphate buffered saline (0.8% NaCl, 0.22% Na_2HPO_4, 0.02% KH_2PO_4, 0.02% KCl, w/w) containing 5% carbonyl iron and 5% gum arabic (Sigma Chemical Co.) was added to 10 ml of whole blood, and the resulting mixture was incubated 45 min at 37° with mixing by inversion (9). The mixture was then diluted with an equal volume of RPMI medium 1640, layered over a solution of phosphate-buffered saline containing 60% Percoll (Pharmacia, Dorval, Que.), and centrifuged (10 min, 400 x g). The lymphocytes were removed from the interface between the Percoll layer and the diluted blood.

Leukemic myeloblasts, supplied by Dr. A. Belch, Cross Cancer Inst., Edmonton, Alb., were purified as follows. Whole blood, obtained as described above from a patient with acute myelogenous leukemia (AML), was diluted with an equal volume of RPMI medium 1640, layered over a solution of phosphate-buffered saline containing 60% Percoll, and centrifuged (10 min, 400 x g). The leukemic myeloblasts were removed from the interface between the diluted blood and the Percoll.

Isolated lymphocytes or leukemic myeloblasts were suspended in RPMI medium 1640 supplemented with 10% fetal bovine serum and were used without further treatment. Cell volumes were determined electrically or radiochemically as described below (section B).

3. Manipulation of population density

The effects of population density on expression of cytodifferentiative markers by BeWo cells were assessed under conditions in which the number of cells per culture varied (Method A) and in which the number of cells per culture remained constant (Method B). Method A: T-25 flasks were innoculated with graded numbers of cells (2.0×10^5 to 10.0×10^5 cells per flask) in a constant volume of growth medium (5 ml/flask). Method B: 60-mm tissue culture dishes were inoculated with the same number of cells (2.0×10^5 cells/dish) in different volumes (0.05 to 5.0 ml) with variable-volume pipetting devices (Eppendorf, Hamburg, West Germany, and Oxford Laboratories, SCP Sciences Division, Dorval Que.) The cells were allowed to attach (3-4 hr) and additional growth medium was then added to a total volume of 5 ml. Six plates were inoculated for each condition; three were trypsinized and used to determine cell numbers and volumes and three were stained with crystal violet to determine the area of the plate covered with cells. The area of the substratum covered with cells was determined by measuring the longest and shortest diameters of the "spot cultures" with a vernier micrometer and using these measurements in the equation of the area of an ellipse ($A = \pi ab$).

Cultures established by Methods A and B were exposed to 1 μM methotrexate as described previously (section A.1) Cell numbers, cell volume distributions, and the area of substrate covered with cells (Method B) were determined at 72 hr. Population densities were calculated using the number of cells present in cultures at 72 hr.

4. Determination of cell viability

a. Colony-forming ability

The reproductive capacity of proliferating and methotrexate-treated BeWo cells was determined in a colony-forming assay. BeWo cultures (50 - 500 cells/60 mm tissue culture dish) were established in drug-free medium, and 24-hr later, culture fluids were replaced with drug-free medium or medium containing 1 μM methotrexate. After 48-hr exposures, culture fluids were replaced with drug-free growth medium containing 100 μM hypoxanthine and 10 μM thymidine. The cultures were left undisturbed for 10 - 14 d at 37° in a 5% CO_2 humidified atmosphere to allow formation of macroscopic colonies. To quantitate colony-forming ability, cultures were stained with a saturated solution of crystal violet in 0.15 M NaCl and macroscopic colonies were scored visually.

b. Vital staining.

For determination of viability by dye exclusion (10), BeWo cultures were disaggregated by trypsinization and cells were suspended in 100 μl of a 0.4% solution of trypan blue (Aldrich Chemical Co., Milwaukee, Wis.) in 0.15 M NaCl. Wet-mounted slides of cell suspensions were prepared immediately, and viable cells were identified by the ability to exclude dye (100-500 cells were scored per assay).

For determination of viability by retention of fluorescein (11), BeWo cultures were disaggregated by trypsinization and cells were suspended in 1 ml of growth medium to which was added 100 μl of a 0.05 mg/ml solution of fluorescein diacetate (Sigma Chemical Co.) in phosphate-buffered (pH 7.4) saline (0.8% NaCl, 0.22% Na_2HPO_4, 0.02% KH_2PO_4, 0.02% KCl, w/w). Cells were incubated at 20° for 15 - 30 min, pelleted by centrifugation (1000 x g, 10 min), resuspended in a small volume of the dye-containing mixture, and wet-mounted slides were prepared. Viable cells were identified by the presence of cytoplasmic fluorescence

(100-500 cells were scored per assay).

The viability of suspended BeWo cells was the same as cells in monolayer cultures and was unaffected by exposure to trypsin for periods of less than 1 hr (data not shown).

c. Thymidine-release assay.

For determination of cell viability by retention of high-molecular weight DNA (12-14), BeWo cultures were established in glass scintillation vials (4 x 10^4 cells/20-ml vial), gassed with 10% CO_2 in air, loosely capped, and incubated at 37° for 24 hr. After 24 hr, culture fluids were replaced with medium containing 20 or 2 nM [*methyl*-^{3}H]thymidine (1.0 or 0.1 μCi/culture, respectively, 50 Ci/mmol). After 24-hr exposures, culture fluids were replaced with drug-free growth medium or growth medium containing 1 μM methotrexate, and the amount of tritium associated with cells was determined at 24-hr intervals as follows. Cultures were rinsed once with 5 ml of cold 0.15 M NaCl and extracted with 0.2 M perchloric acid (2 ml) for 20 min at 4°. The resulting precipitates were air-dried overnight and solubilized with 2 ml of 0.3 N KOH. A xylene-detergent scintillation fluor (8 ml/vial) was added (15), and the radioactive content of samples was determined by liquid scintillation counting (LS 7500, LS230, or LS-3133T liquid scintillation counters, Beckman Instruments, Inc., Irvine, Calif.).

To determine if radioactivity released by dead cells was reutilized, untreated and methotrexate-treated BeWo cultures were incubated in growth medium containing [^{3}H]thymidine-labeled cells that were killed by freeze-thawing as follows. A single BeWo culture was established with 2 x 10^6 cells/T-75 flask, and after 24 hr, culture fluids were replaced with growth medium containing 100 μCi of [^{3}H]thymidine (0.2 μM, 20 Ci/mmole). After a 24-hr exposure, culture fluids were removed by aspiration, and cultures were washed 3 times with medium (10 ml/wash). Cells were disaggregated with trypsin-EDTA, pelleted by centrifugation, and resuspended in medium at 10^5 cells per ml. The cell suspensions were frozen in a dry ice-acetone bath, thawed, and warmed to 37°.

B. Cell Volume Measurements

1. Electrical

Electronically edited (16) cell volume distributions were obtained with a Model Zf Coulter Counter (100-μm aperture tube) in conjunction with a 100-channel particle size analyzer, Channelyzer II, Coulter Electronics Inc., interfaced with an Apple II plus computer, Apple Computer Inc., Cupertino, Calif. The channelyzer was calibrated with polystyrene microspheres of 10.08 or 9.69 μm diameter obtained from Coulter Electronics Inc. All volume distributions were accumulated at a coincidence rate of less than 5% until the modal peak totaled 1000 cells. Volume distributions were analyzed with software obtained from Coulter Electronics Inc. A shape factor of 3/2, corresponding to a spherical non-conducting particle, was used for all determinations of cell volume. Mean, median, and modal cell volumes for each volume distribution were calculated, and, unless otherwise indicated, values reported were the average of several separate determinations of modal cell volume.[3]

The relationship between voltage pulse height and particle volume was investigated with microspheres of known diameters obtained from Coulter Electronics, Inc. (5, 10, 9.69, 10.08, 10.14 μm), Duke Standards, Palo Alto, Calif. (9.54 ± 0.15 μm), 3M Company, Inc., Nuclear Products Division, St. Paul, Minn. (15 ± 5 μm) and Sigma Chemical Co. (25.7 ± 5.8 μm).

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[3] To avoid confusion, the following definitions have been used for reporting all measurements of cell volume. Mean, median, and modal volume refer to values determined from a *single* volume histogram, and the standard deviation (S.D.) represents the dispersion around the mean of the distribution. The arithmetic mean of 3 or more separate volume determinations was referred to as the *average* modal or mean volume, and the S.D. of the average represents the interassay dispersion in determination of mean or modal cell volumes.

2. Radiochemical

Cell-associated water was determined using a centrifugal method (17). Cultured cells were suspended in the appropriate growth medium and blood cells were suspended in RPMI medium 1640 supplemented with 10% fetal bovine serum. Triplicate (duplicate for L1210/C2 cells) assay mixtures were prepared in 1.5-ml polypropylene microcentrifuge tubes (Bio-Rad Laboratories, Mississauga, Ont.). Added first was 150 μl of oil (84.9 parts Dow Corning 550 silicone oil and 15.1 parts Fisher 0-119 light paraffin oil; specific gravity, 1.03 g/ml) upon which was layered 100 μl of growth medium containing [^{3}H]water (2 μCi/ml) or [^{14}C]sucrose (10 μCi/ml). Reactions were initiated by addition of 100 μl of growth medium containing 1 x 10^6 to 3 x 10^6 cells and were ended immediately thereafter by centrifugation for 30 sec (Eppendorf Model 3200 microcentrifuge, Eppendorf) The media portions of the incubation mixtures were removed by suction, and tubes were rinsed with 1.0 ml water. The rinse and most of the oil were removed by suction leaving the pellet and a small portion of the oil in each tube. For assay of the pellet content of radioactivity, 200 μl of 5% Triton X-100 was added to each tube, and after incubating overnight, the tube contents and tubes were transferred to counting vials and 10 ml of scintillation fluor was added (15). Radioactivity was determined by liquid scintillation counting.

3. Optical

BeWo cultures were harvested by trypsinization and the disaggregated cells were suspended in 0.15 M NaCl. Polystyrene beads of a known diameter (9.54 μm, Duke Standards, Inc.) were added to the suspensions of BeWo cells and wet mounts were used for phase-contrast photomicroscopy. Photomicrographs were taken with a camera mounted on a Zeiss Universal microscope (Carl Zeiss (Canada) Ltd.), and the diameters of beads and the major and minor diameters of BeWo cells were measured directly from photomicrographs with a vernier micrometer.

C. Incorporation of Radioactive Precursors into Macromolecules

1. Thymidine and deoxyuridine

The apparent intracellular rate of thymidylate synthesis was assessed by measuring utilization of deoxyuridine and thymidine from culture fluids. BeWo cultures were established in glass scintillation vials (4.0 x 10^4 cells/20-ml vial) and left undisturbed at 37° for 48 hr. Exposures to 6.7 nM [*methyl*-^{3}H]thymidine or 22 nM [6-^{3}H]deoxyuridine were initiated by adding 1 ml of growth medium containing 1 μCi radioactivity to the existing culture fluids (final volume 3 ml) and incorporations were ended after 10 min by aspiration of culture fluids and acid precipitation with 0.2 M perchloric acid. Cultures were extracted for 20 min at 4°, acid was removed by aspiration, and vials were inverted and allowed to air-dry overnight. Acid-precipitable materials were resolubilized with 0.3 N KOH (2 ml/vial), 10 ml of scintillation fluor (15) was added per vial, and radioactivity was determined by liquid scintillation counting. The rates of incorporation of [*methyl*-^{3}H]thymidine or [6-^{3}H]deoxyuridine into acid-precipitable material were constant at least 30 min. The rates of incorporation of thymidine and deoxyuridine by drug-free cultures were not significantly different ($p>0.05$) over the 2-hr exposures, and because [*methyl*-^{3}H]thymidine or [6-^{3}H]deoxyuridine were stable in growth medium (data not shown), any changes in the rates of incorporation of either precursor were not related to changes in their extracellular specific activity.

2. Utilization of [^{14}C]formate

BeWo cultures were established with 2 x 10^5 cells per 60 mm tissue culture dish and were incubated in drug-free growth medium at 37° for 48 hr. Cultures were then "pre-incubated" with growth medium supplemented with 10% dialyzed fetal bovine serum with or without 1 μM methotrexate for 1 hr. Immediately thereafter, intervals of incorporation were initiated by adding 250 μl of growth medium containing 41.7 μCi [^{14}C]formate to each culture; the final concentration of formate was 0.17 mM (18.5 μCi/ml). Intervals of incorporation were ended by aspiration of culture fluids at graded time intervals; cultures were rinsed with 5 ml of cold 0.15 M NaCl and were extracted with 2.5 ml of 0.2 M perchloric acid at 4° for 20 min. The culture flasks were rinsed with an additional 2.5 ml of ice-cold 0.2 M perchloric acid, inverted, and allowed to air-dry overnight. DNA, RNA, and protein were separated as follows (18). The precipitates in culture flasks were completely resolubilized by incubation with 2 ml of 0.3 M NaOH at 37° for 60 min. This procedure hydrolyzes RNA to acid-soluble fragments, whereas DNA and protein remain acid-precipitable. Alkaline solutions were acidified with an equal volume of 0.4 M perchloric acid, cooled, and centrifuged (1000 x g, 10 min) at 4°. The supernatants (RNA containing fractions) were transferred to scintillation vials and 16 ml of fluor (15) was added to each vial. Precipitates remaining after alkaline hydrolysis (containing DNA and protein) were treated with 0.5 M perchloric acid (2 ml) at 70° for 25 min. This procedure hydrolyzes DNA to acid-soluble fragments, whereas protein remains acid-precipitable. Acidified solutions were cooled to 4°, centrifuged (1000 x g, 10 min), and the supernatants (DNA containing fractions) were transferred to scintillation vials and 8 ml of fluor (15) was added to each vial. Acid-insoluble materials remaining after acid hydrolysis (protein-containing fractions) were resolubilized with 2 ml of 0.3 M NaOH (4 hr incubation at 37°), transferred to scintillation vials, and 8 ml of fluor was added to each vial (15). Radioactivity was determined by liquid scintillation counting.

3. Adenine and hypoxanthine incorporation into RNA

Relative rates of purine salvage were assessed by measuring incorporation of [8-^{3}H]adenine or [8-^{14}C]hypoxanthine into RNA by variously treated cultures of BeWo cells. After incorporation periods of up to 60 min, cultures were rinsed with 5 ml of ice-cold 0.15 M NaCl and extracted with 5 ml of 0.2 M perchloric acid at 4° for 20 min. RNA was separated from DNA by the Fleck and Munroe modification of the Schmidt-Thannhouser procedure (19). Perchloric acid-insoluble material was solubilized by incubating cultures at 37° with 2 ml of 0.3 M KOH for 1 hr. The resulting solutions were transferred to test tubes, chilled to 4°, acidified with equal volumes of 0.4 M perchloric acid, and after incubating at 4° for 20 min, centrifuged. The RNA-containing fractions (supernatants) were transfered to counting vials and 15 ml of scintillation fluor (15) was added to each vial. Radioactivity was determined by liquid scintillation counting.

D. Hypoxanthine Uptake

Rates of hypoxanthine uptake in the presence or absence of 10 μM dipyridamole were measured at 37° using a centrifugal method (13). BeWo cultures for experimental use were established with 2 x 10^6 cells/T-150 flask and harvested 5 d later. Cells were rinsed once with 20 ml of 0.15 M NaCl at 37°, trypsinized, suspended in growth medium, pelleted by centrifugation, and resuspended in growth medium at a density of 8.56 x 10^5 cells/ml (100 ml total in a 250 ml glass bottle). Cell suspensions were gassed with 10% CO_2 in air, tightly capped, and incubated for 1 hr with rotary shaking (175 rpm, Model G2 gyrotory shaker, New Brunswick Sci. Co., Edison, N.J.) The viability of cells in this suspension was > 95% as determined by the fluorescein diacetate method (section A.4). Before rates of uptake were assessed, cells were pelleted by centrifugation and resuspended at a density of 1 x 10^6 cells/100 μl in medium with or without 10 μM dipyridamole. Dipyridamole-treated cells were preincubated for 3 min before intervals of uptake were initiated. Single assay mixtures were prepared in 1.5 ml polypropylene microcentrifuge tubes (Bio-Rad Laboratories). Added first was 150 μl of oil (84.9 parts Dow Corning 550 silicone oil and 15.1 parts Fisher 0-119 light paraffin oil; specific gravity, 1.03 g/ml) upon which was layered 100 μl of growth medium containing [^{3}H]hypoxanthine (10.7 μM, 203 cpm/pmol). Uptake intervals were initiated by adding 100 μl of growth medium containing 1.0 x 10^6 cells to each tube. Intervals of uptake were ended by centrifugation for 30 sec (Eppendorf Model 3200 centrifuge). Metabolism of the permeant during the intervals of incorporation was not assessed. Tubes were treated as described previously (section B.2) and the [^{3}H]content of cell pellets was determined by liquid scintillation counting.

In parallel experiments, intracellular water volume was determined as described previously (section B.2).

E. Measurement of DNA, RNA, and Protein Content

1. DNA

The DNA content of BeWo cells was quantitated by flow cytometric analysis of ethanol-fixed, mithramycin-stained, cell suspensions (20). Proliferating or methotrexate-treated cultures were disaggregated by trypsinization, and cells were suspended in growth medium at 4°, pelleted by centrifugation, and resuspended in cold 0.15 M NaCl (1 x 10^6 cells/ml). Cells were fixed at 4° by slowly adding 5 ml of cold 70% ethanol per 1 ml cell suspension. If not used immediately, fixed cells could be stored at 7° for up to 1 week without changes in DNA histograms. In some cases, chick erythrocytes were added as an internal standard (20) to the fixed cell suspensions 30 min prior to the staining procedure. Fixed cells (with or without internal standards) were pelleted by centrifugation (1000 x g, 10 min) resuspended in the staining solution (100 μg/ml mithramycin; Sigma Chemical Co., 0.1 M NaCl, 15 mM $MgCl_2$, 0.1 M tris-HCL, pH 7.6) at 10^6 cells/ml and incubated in the dark for 30 - 45 min. All analyses of cellular fluoresence were completed within 3 hr of suspension of cells in the staining solution.

Mithramycin preferentially binds to guanine-cytidine regions of helical DNA, but does not bind to RNA. Binding of mithramycin to DNA results in a slight blue shift of the major emmision peak (approximately 10 nM) and a 10-fold increase in fluorescent emmission intensity (20). The relative DNA content of BeWo cells was determined by flow cytometric analysis of fluorescence of wavelengths greater than 570 nm after excitation with incident light at a wavelength of 457 nm (argon ion laser, 200 mW) with a Coulter Electronics EPICS V flow cytometer. Emitted fluoresence was analyzed after passing through a 515 nm blocking filter, a 560 nm dichroic mirror, and a 570 nm longpass barrier filter. If measured, forward angle and 90° light scatter by BeWo cells were analyzed after passing through a 488 dichroic mirror.

The relative number of cells in compartments of the cell cycle were determined with software (PARA2) obtained from Coulter Electronics on a Terak 8510 computer (21). DNA distributions that were not analyzed with the PARA2 program were smoothed and translocated with the PARA1 program (Coulter Electronics Inc.).

2. RNA

RNA was determined with the Fleck and Munroe modification of the Schmidt-Thannhauser procedure for the determination of nucleic acid (19). Cultures were rinsed with 5 ml of ice-cold 0.15 NaCl, extracted at 4° for 20 min with 5 ml of 0.2 M perchloric acid, and air-dried overnight. The precipitates were hydrolyzed with 0.3 M KOH (2 ml/flask) for 1 hr at 37°, and acid-precipitable material was removed and discarded by adding an equal volume of 0.4 M perchloric acid, incubating 20 min at 4°, and centrifuging (1000 x g, 10 min). The supernatants (RNA-containing fractions) were diluted with equal volumes of water, and the concentration of RNA was determined by absorbance at 260 nm.

3. Protein

The protein content of cell cultures was determined with the Oyama and Eagle modification of the Lowry procedure as follows (22). Cultures were rinsed once with 5 ml of 0.15 M NaCl and incubated with 2 ml of Lowry solution "C" prepared just before use by mixing 100 ml of solution "A" (2% Na_2CO_3 and 0.4% NaOH w/w in water) with 2 ml of solution "B" (1% $CuSO_4 \cdot 5\ H_2O$ and 2.7% sodium potassium tartrate, w/w in water). After incubation overnight at room temperature, 100 μl of water and 0.2 ml of 1 N Folin and Ciocalteau reagent (Fisher Scientific, Co.) were added, and the flasks were incubated at room temperature for 30 min. The protein standard (range was 5 - 100 μg protein/assay) was bovine serum albumin (Fraction V, Sigma Chemical Co.). Absorbance of experimental samples and protein standards was measured at 660 nm with a Beckman Model 35 spectrophotometer (Beckman Instruments, Inc.).

F. High Performance Liquid Chromatography

1. Purine content of fetal bovine serum

Analyses of the purine content of fetal bovine serum were performed by reversed-phase high performance liquid chromatography. Separations were carried out by isocratic elution on a Whatman Partisil PXS 5/25 ODS-3 column (Whatman, Inc., Clifton, N.J.). The chromatography system consisted of a Spectra-Physics pump (Model 740P, Santa Clara, Calif.) operated at constant flow by a Chromatronix pump controller (Spectra-Physics, Model 740-C), a Waters injection loop (Model U6K, Waters Associates, Milford, Mass.), a Pharmacia UV detector (Model 100, 254 nm), an Autolab Minigrator (Spectra-Physics), and a Varian A-25 strip chart recorder (Varion Aerograph, Walnut Creek, Calif.). Purine bases were eluted with 50 mM phosphate, pH 4.6, at a flow rate of 1 ml/min. This chromatography system afforded excellent resolution of adenine, guanine, hypoxanthine, xanthine, and uric acid and separated the purine bases from purine nucleosides (adenosine, guanosine, and inosine). Hypoxanthine and xanthine were identified by retention time, cochromatography with added standards, and enzymatic shift to uric acid with xanthine oxidase. Other bases were identified by retention time and cochromatography with added standards. Nucleosides were isocratically eluted with 50 mM phosphate:methanol (94:6), pH 4.6, at a flow rate of 1 ml/min. This procedure resolved adenosine, guanosine, inosine, and the purine bases. Analyses of guanosine and inosine were performed following fractionation of 1 ml of serum on a boronate gel (Affi-Gel 601, Bio-Rad Laboratories Inc.) by elution with 0.1 M formic acid (23). Effluents were collected, lyophilized (Virtis lyophilizer, Virtis Co., Gardiner, N.Y.), and resuspended in

250 μl of distilled water. Nucleosides were identified by retention time and cochromatography with added standards. Concentrations of bases and nucleosides were determined by comparison of either peak height or area with that of authentic standards. Protein was removed from serum samples by centrifugation at 900 x g through Amicon CF25 centriflo membrane cones (Amicon Corp., Lexington, Mass.).

2. Methotrexate concentration in culture fluids

Samples of growth medium used for the determination of methotrexate were prepared for chromatography using a preliminary ion-exchange procedure. Ten-ml samples (obtained by combining media from 2 replicate cultures) were applied to 0.7 x 10-cm columns of Dowex-1 (1X180, Sigma Chemical Co.) mounted on a 'Baker'-10 vacuum manifold (J.T. Baker Chemical Co., Phillipsburg, N.J.). The columns were washed, under vacuum, with 25 ml of distilled water and 25 ml of 0.2 M acetic acid and the effluents were discarded. Methotrexate was eluted with 10 ml of 2 M acetic acid. The methotrexate-containing effluent was collected, lyophilized (Virtis lyophilizer, Virtis, Co.) and resuspended in 250 μl of distilled water.

Analyses of the concentration of methotrexate in samples of growth media were performed by reversed-phase high performance liquid chromatography. Chromatographic separations were carried out by isocratic elution of a 3.9-mm (internal diameter) x 30-cm Waters μBondapak C_{18} column with a mobile phase consisting of acetate (0.2 M, pH 5.5) buffer:methanol:acetonitrile (85.3:3.4:6.3) at a flow rate of 1.5 ml/min (24). This chromatographic system, which separates methotrexate from its metabolites 7-hydroxymethotrexate and 2,4-diamino-10-methyl-pteroic acid (24), separated methotrexate from UV-absorbing material present in methotrexate-free growth medium. Three samples (each pooled from two replicate cultures) of medium were assayed per condition. The methotrexate peak was identified by retention time and cochromatography with authentic methotrexate. Methotrexate was quantitated by integrating the area under the UV absorption peaks. The methotrexate calibration curve was linear from 1.25 to 5 nmol, a concentration range that bracketed the amount of methotrexate in the samples injected onto the column. The coefficient of variation for the retention time of methotrexate standards was 3.9% and for areas of a known methotrexate concentration was 4.9% (n=10).

3. Purification of radiochemicals

[8-^{3}H]Adenine and [8-^{14}C]hypoxanthine were repurified by reversed-phase high performance liquid chromatography by isocratic elution of a Waters μBondapak C_{18} column with distilled water at a flow rate of 1 ml/min. This chromatography system completely resolved adenine from hypoxanthine. [*methyl*-^{3}H]Thymidine and [6-^{3}H]deoxyuridine were repurified by isocratic elution of a Waters μBondapak C_{18} column with methanol:water (5:95) at a flow rate of 1 ml/min. After purification, all radiochemicals were dried by rotary evaporation at 37° under vacuum, resuspended in distilled water, and stored at 2° for a maximum of 7 d before use.

G. Alkaline Phosphatase

1. Histochemical localization

Alkaline phosphatase activity was histochemically localized by hydrolysis of a chromogenic substrate (25). The substrate solution was prepared by dissolving 10 mg of 3-hydroxy-2-napthoic acid 2,4-dimethylanilide phosphate (AS-MX phosphate, Sigma Chemical Co.) in 0.25 ml dimethylformamide (Aldrich Chemical Co.). This solution was diluted with 25 ml distilled water and an additional 25 ml of 0.1 M 2-amino-2-methyl-1-propanol in water, pH 10, was added. Immediately before use, 30 mg of 5-chloro-2-toluenediazonium chloride hemizinc chloride (Fast Red TR salt, Sigma Chemical Co.) was added to the substrate solution and filtered (Whatman qualitative paper, grade 1, Whatman Inc, Clifton, N.J.). To stain cells, cultures were rinsed 3 times with cold 0.15 M NaCl (5 ml/wash), fixed at 4° with a 10% solution of formaldehyde (37%, w/w) in absolute methanol, and incubated at room temperature with the substrate solution for 15-20 min. Intervals of incubation were ended by aspirating the substrate solution and rinsing once with 10 ml of cold 0.15 M NaCl, and alkaline phosphatase-positive cells (red-stained cytoplasm) were identified by phase-contrast microscopy.

2. Heat-stable alkaline phosphatase assay

BeWo cells possess heat-stable and heat-labile forms of alkaline phosphatase. The phosphatase activity remaining after incubating extracts at 60° (5-60 min) represents the alkaline phophatase isoenzyme expressed by term placenta (26). Cells were harvested by scraping cell monolayers (Costar disposable cell scraper, Costar, Cambridge, Mass.) into 2 ml of 0.15 M NaCl at 4°; and resulting mixtures were stored at -20°. To obtain extracts for assay of heat-stable alkaline phosphatase activity, the cells were thawed, suspended by vortexing, sonicated at 4° (3 x 15 s at 3 amperes with a Branson Instruments model LG-75 sonifier, Stamford, Conn.), centrifuged (34,800 x g, 50 min); the pellets were discarded and the supernatants were heated at 60° for 5-15 min. Alkaline phosphatase was assayed by the method of Lowry (27). Reactions were initiated by mixing 100 μl of cell extracts and 250 μl of substrate solution (8 mM *p*-nitrophenyl phosphate and 2 mM $MgCl_2$ in 0.5 M 2-amino-2-methyl-1-propanol, pH 10), and after incubating for 30 min at 37°, reactions were stopped by adding 0.75 ml of 0.25 M NaOH. The amount of *p*-nitrophenol produced during the assay period was determined by measuring the absorbance of the reaction mixture at 400 nm. The activity of heat-stable alkaline phosphatase was unaffected by repeated freezing and thawing over a period of at least 4 weeks (data not shown). The protein content of extracts was determined with the Coomassie Brilliant Blue G-250 dye binding assay (28). The crude extract (100 - 300 μl) was added directly to 3 ml of dye reagent (1x, Bio-Rad Laboratories, Inc.). Bovine serum albumin (Fraction V, Sigma Chemical Co.) was used as the protein standard (range 5-100 μg/assay).

H. Statistical Analysis

The dispersion of sample means was reported as the sample standard deviation (S.D.). To determine the mean ± S.D. of two series of experimentally determined values, a program function (X°.÷Y) of the MTS\APL computer language (a version of VS APL, IBM Corporation, Armonk, N.Y., modified to function under the Michigan terminal system operated on an Amdahl 5860 computer at the University of Alberta, Edmonton, Alberta) was used to calculate values for every combination of X and Y (29), and the mean and S.D. were calculated from these values.

Student's t-test, which assumes normal populations with equal standard deviations, was used to determine if sample means were significantly different at the 95% confidence level (30).

I. Chemicals

Fetal bovine sera used in growth media and for analysis of purine content were obtained from Grand Island Biologic Co.; Flow Laboratories; M.A. Bioproducts, Mississauaga, Ont.; and Animal Health Laboratory, Toronto, Ont.

Methotrexate was obtained from Sigma Chemical Co. and Lederle Laboratories, Pearl River, N.Y. and fluorodeoxyuridine was obtained from Sigma Chemical Co. Dipyridamole was a gift to Dr..A. R. P. Paterson from Boehringer Ingelheim (Canada), Burlington, Ont.

High performance liquid chromatography-grade methanol, acetonitrile, and potassium phosphate were purchased from Fisher Scientific Corp. Xanthine oxidase (grade III from buttermilk), purine bases, and nucleosides were obtained from Sigma Chemical Co.

[8-^{3}H]Adenine (10 Ci/mmol) was purchased from New England Nuclear (NEN (Canada) Lachine, Que.). [8-^{3}H]Hypoxanthine (10 Ci/mmol), [8-^{14}C]hypoxanthine (57 mCi/mmol), [*methyl*-^{3}H]thymidine (50 Ci/mmol), [6-^{3}H]deoxyuridine (18 Ci/mmol), and [^{14}C]formate (57 mCi/mmol), were purchased from Moravek Biochemicals Inc., Brea, Calif. [^{3}H]Water (100 mCi/ml) and [*U*-^{14}C]sucrose (250 mCi/ml) were purchased from ICN Chemical and Radioisotope Division, Irvine, Calif.

Other chemicals were obtained from commercial sources.

J. References

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V. Biological Effects of Methotrexate on BeWo cells

A. Introduction

BeWo cells, although derived from a malignant choriocarcinoma, possess biological and biochemical characteristics associated with the trophoblast of *in utero* placenta. BeWo cells synthesize and secrete chorionic gonadotropin, placental lactogen, steroid hormones, and placental alkaline phosphatase (1, 2), markers of normal syncytiotrophoblastic function (3-6). In addition, proliferating BeWo cells are morphologically similar to normal cytotrophoblast (7-11). A second cell type is also present in proliferating BeWo cultures at a low frequency (1 - 4% of the total population). These cells are larger than CTL cells, often multinucleated (10, 11), and ultrastructurally similar to the syncytiotrophoblast of *in utero* placenta (11).

Morphological and biochemical changes initiated by methotrexate indicate that BeWo CTL cells can be induced to differentiate to the STL phenotype. In cultures exposed to methotrexate for 2 - 4 days, 80 - 90% of the cells are morphologically indistinguishable from spontaneously occuring STL cells present in proliferating BeWo cultures (11). Methotrexate also stimulates BeWo cell expression of chorionic gonadotropin and placental alkaline phosphatase (1).

Although BeWo cells were derived from a metastatic choriocarcinoma obtained from a patient that developed resistance to methotrexate chemotherapy (9, 12) and have been described as "methotrexate-resistant" in the literature (13, 14), the degree or biochemical mechanism(s) of resistance to the toxic effects of methotrexate have not been described. Furthermore, although it has been suggested that methotrexate induces *nontoxic* cytodifferentiative changes in BeWo cells (11), the viability of drug-treated cells has not been previously determined in a colony-forming assay. To assess the relationships between differentiation and sensitivity to the lethal effects of methotrexate, experiments were undertaken to quantitate the antiproliferative, toxic, and differentiative effects of methotrexate on BeWo cells.

Methotrexate-induced morphological differentiation from the CTL-to-STL phenotype (11) and expression of chorionic gonadotropin and placental alkaline phosphatase (1) have been well characterized, but under dissimilar conditions. In this work, quantitative markers of the CTL-to-STL differentiative response were developed and the time- and concentration-relationships for methotrexate's effects on BeWo cell proliferation, viability, and expression of syncytiotrophoblastic markers were determined under identical culture conditions. Since fetal bovine serum is known to contain endogenous metabolites that modulate methotrexate toxicity, the ability of dialyzed and untreated serum to support expression of cytodifferentiative markers were investigated.

Under culture conditions permissive for the CTL-to-STL cytodifferentiative response, the time courses of appearance and concentration-effect relationships of differentiative markers during continous exposure to methotrexate were similar. Inhibition of proliferation and expression of syncytiotrophoblastic markers had similar concentration-effect relationships, whereas colony-forming ability was unaffected over the range of methotrexate concentrations tested.

During these investigations it became apparent that culture conditions profoundly altered expression of markers of the CTL-to-STL differentiative response. Methotrexate-induced increases in cell mass, but not expression of placental alkaline phosphatase or differentiation to the STL phenotype, were dependent on a dialyzable factor present in serum. In addition, an increase in the number of cells per culture reduced expression of syncytiotrophoblastic markers during exposure to methotrexate; hence, all BeWo cultures used during the investigations reported in this chapter were established at an initial population density of $<1.3 \times 10^4$ cells/cm^2. Experiments undertaken to identify the factor lost during dialysis and to investigate the basis of inhibition of the differentiative response observed in crowded cultures are presented in Chapters VI and VIII, respectively.

B. Results

1. Antiproliferative and cytotoxic effects of methotrexate

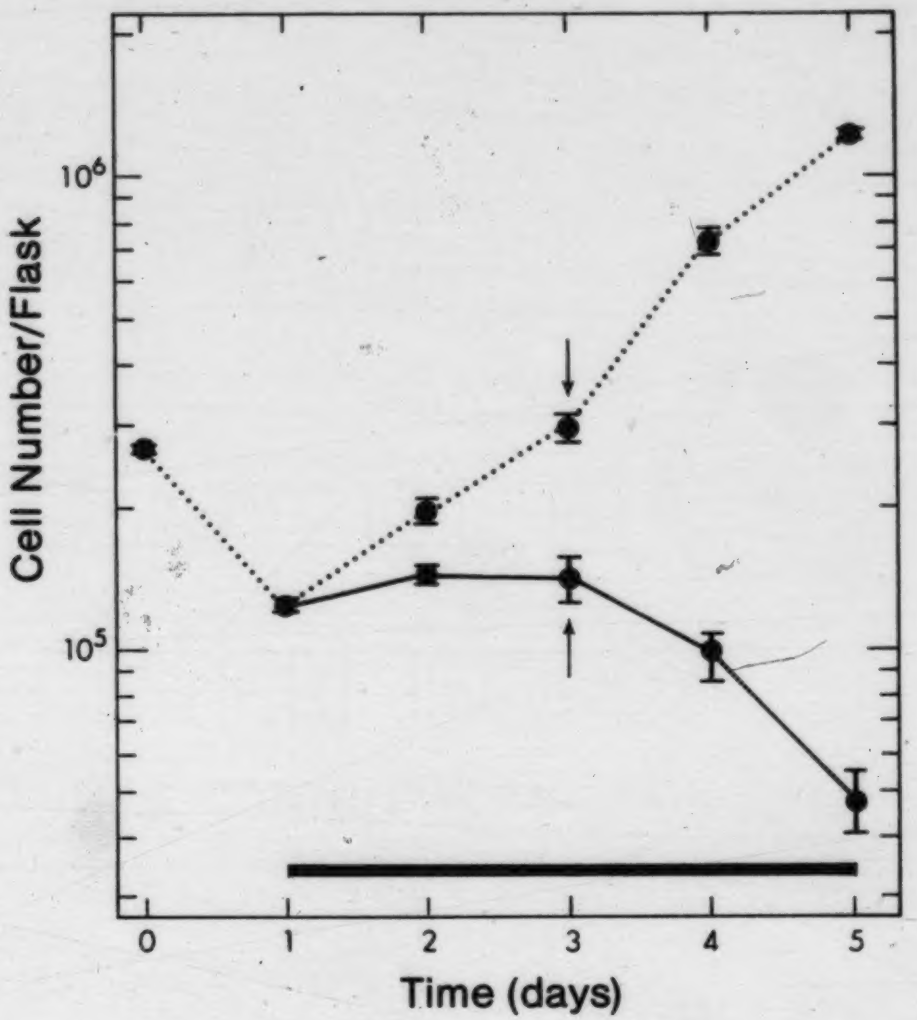
a. Inhibition of proliferation

Before undertaking studies with methotrexate, conditions for proliferation of BeWo cells were established. A growth curve, representative of those obtained for BeWo cultures established under the conditions employed throughout this work, is presented in Figure 5. Following an initial lag period of 24 hr, a complete change of growth medium every 48 hr was sufficient to support continued proliferation of BeWo cultures for at least 4 mean population doublings. In subsequent experiments, cells were exposed to methotrexate with a complete change of growth medium 24 hr after cultures were initiated to allow recovery from the adverse affects of subculturing. It is evident from the results presented in Figure 5 that continous exposure to 1 μM methotrexate completely inhibited proliferation of BeWo cells. The cell number in drug-treated cultures did not increase after exposure to methotrexate and declined after 48 hr-exposures to drug. The decline in cell numbers and the presence of debris in culture fluids following prolonged exposure ($>$ 48 hr) to methotrexate suggested significant loss of viability, but formation of syncytia could also explain decreased cell number.

These results differ from those of other investigators, who have reported that prolonged exposures to 1 μM methotrexate (3-5 d) did not result in significant losses of cells from cultures (11, 13). It is possible that this discrepancy resulted from a change in drug sensitivity from drift in culture or variations in the proliferative state of the cultures. Previous investigators used cultures that were established at an initial cell density 10-fold greater than the cultures of the experiment presented in Figure 5, and the mean population doubling times of cultures at the higher population density were significantly slower (ref. 11; 58±19 hr, and ref. 13; 50±12 hr). It is well established that slowly growing cells are relatively resistant to the cytotoxic effects of methotrexate (15-17).

An IC_{50} value of 50 nM was obtained for inhibition of BeWo cell proliferation by methotrexate in medium supplemented with dialyzed fetal bovine serum (Figure 6). Thus, BeWo cells were highly sensitive to the antiproliferative effects of methotrexate even though the BeWo cell line was derived from a choriocarcinoma that was clinically resistant to methotrexate chemotherapy.

Figure 5. Effects of methotrexate on proliferation of BeWo cells in growth medium supplemented with 10% fetal bovine serum. Cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 and 72 hr, culture fluids were replaced with drug-free medium (●·●) or medium containing 1 μM methotrexate (●—●) (Materials and Methods, section A). At 24-hr intervals, cells were disaggregated by trypsinization, suspended in 0.15 M NaCl, and counted with an electrical particle counter. The shaded bar indicates exposure to methotrexate, and each point represents the cell number (mean ± S.D, n=3) from a representative experiment. The mean population doubling times (±S.D., n=37) of BeWo cultures, under the experimental conditions of this representative growth curve, maintained in medium supplemented with 10% undialyzed serum (23.4 ± 7.3 hr) or medium supplemented with 10% dialyzed serum (24.3 ± 6.1 hr) were the same ($p>0.05$). After 48-hr exposures to methotrexate, cell numbers in drug-treated cultures were the same ($p>0.05$, n=4 separate triplicate experiments) in growth media supplemented with untreated or dialyzed serum (data not shown). In subsequent experiments, expression of markers of the CTL-to-STL differentiative response by proliferating and methotrexate-treated cells were compared 72 hr after cultures were initiated (indicated by the arrows in this graph).


Cell Number/Flask
10^6
10^5
0
1
2
3
4
5
Time (days)

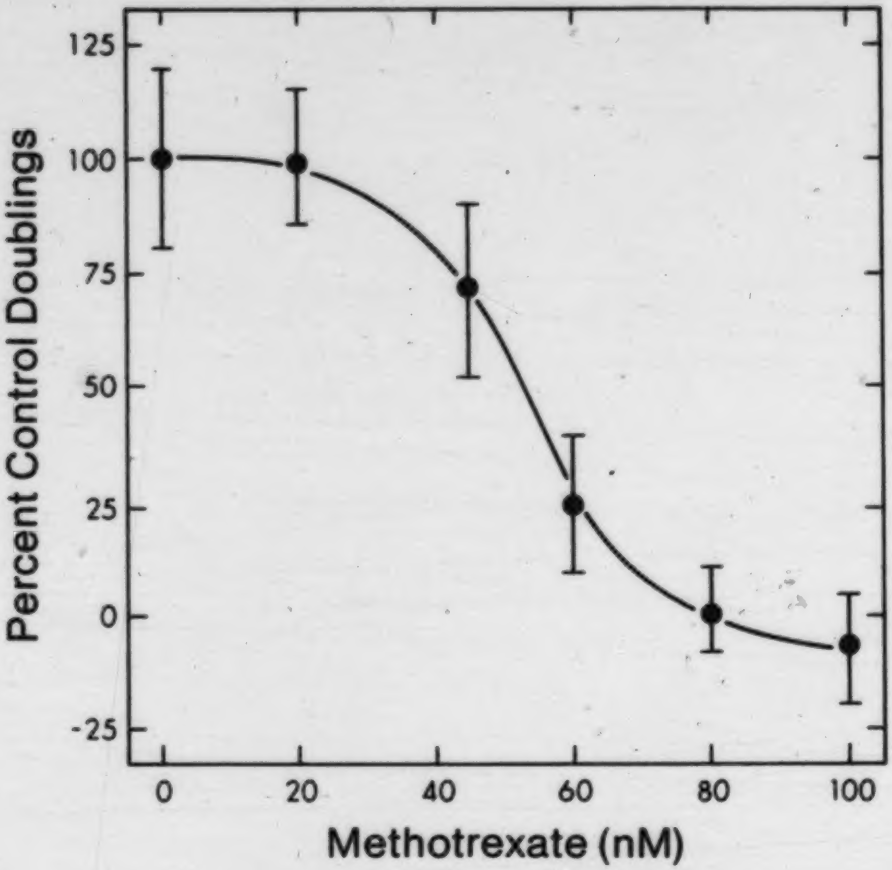


Figure 6. Determination of the IC_{50} value for inhibition of BeWo cell proliferation by methotrexate. BeWo cultures were established with 2 x 10^5 cells/T-25 flask and, after 24 hr, culture fluids were replaced with drug-free growth medium (dFBS) or medium (dFBS) containing methotrexate at the concentrations indicated. At 24 and 72 hr, cell numbers were determined, and symbols represent the number of cell doublings (mean ± S.D., n=4) of variously treated cultures expressed as a percentage of doublings in the absence of drug (1.9±0.4 doublings; mean population doubling time of 26.1±6.8 hr).

b. Viability of BeWo cells

The decline in cell numbers and presence of debris in culture fluids following exposure to methotrexate (> 48 hr) suggested significant loss of viability, but it was also possible that cell number decreased because of formation of syncytia. To determine if decreased cell numbers following exposure to methotrexate resulted from loss of cells from the cultures or cell fusion, the amount of acid-precipitable DNA remaining in cultures was assessed with a thymidine-labeling radioassay technique. Cells were prelabeled with [^{3}H]thymidine and the amount of radioactivity associated with untreated and methotrexate-treated cells was determined at various intervals. The loss of radiolabeled thymidine from prelabled monolayer cultures has been used previously to quantitate cell death (18-21).

Before the effects of methotrexate on retention of radiolabeled DNA was assessed, proliferation rates of BeWo cells following exposure to [^{3}H]thymidine were determined. It is evident from the data shown in Table 2 that exposure of cultures to 0.1 μCi [^{3}H]thymidine was without effect, whereas exposure to 1 μCi [^{3}H]thymidine inhibited BeWo cell proliferation. In subsequent experiments, cells were radiolabeled by 24-hr exposures to 0.1 μCi [^{3}H] or 0.1 μCi [^{14}C]thymidine per culture.

Table 2

Effects of $[^3H]$Thymidine on BeWo Cell Proliferation

	Cell Number/Vial (x 10^{-4})		
Time (hr)	Untreated	0.1 μCi/vial	1.0 μCi/vial
0	2.74 ± 0.13	2.68 ± 0.17	2.78 ± 0.41
24	4.64 ± 0.46	3.21 ± 0.16	4.55 ± 0.22
48	13.0 ± 1.2	13.5 ± 1.7	6.13 ± 1.30
72	23.8 ± 1.3	32.2 ± 2.6	3.87 ± 0.52

BeWo cultures were established with 4 x 10^4 cells/20 ml glass scintillation vial, and after 24 hr, culture fluids were replaced with growth medium (1 ml) containing 20 nM (1.0 μCi/vial) or 2 nm (0.1 μCi/vial) $[^3H]$thymidine (50 Ci/mmol). After 24-hr exposures, culture fluids were replaced with drug-free medium (2 ml), and cell numbers (mean±S.D., n=3) were determined with an electrical particle counter. Thereafter, cell numbers were determined at 24-hr intervals, and after 48 hr, culture fluids were replaced. The mean population doubling times of untreated (25.0±6.0 hr) and 0.1 μCi $[^3H]$thymidine-treated cultures (24.8±7.3 hr) were the same ($p>0.05$).

It is evident from the results presented in Table 3 that exposure to methotrexate did not cause significant loss of [^{3}H]radiolabled DNA during moderate exposures (24-72 hr), but continued exposure (6 d) led to significant decreases in the amount of radioactivity associated with cells. Qualitatively similar results (not shown) were observed with [^{14}C]thymidine-treated BeWo cultures. Thus, decreased cell numbers during continous and prolonged exposure to methotrexate apparently resulted from cell death and loss of cellular material from the cultures.

If BeWo cells reutilized significant amounts of radioactive thymidine released from dead cells, loss of radiolabeled DNA would underestimate cell death. To determine if reutilization of radiolabeled DNA occured in the experiment presented in Table 3, BeWo cultures were exposed to growth medium containing freeze-thawed cells that had been previously labeled with [^{3}H]thymidine. The amounts of tritium from prelabeled freeze-thawed cells associated with BeWo cultures after 48-hr exposures was approximately 20% of the total radioactivity available from culture fluids (Table 4) Thus, loss of radioactivity from methotrexate-treated BeWo cultures probably underestimated loss of labeled DNA by approximately 20% in the experiment of Table 3.

Table 3

Effects of Methotrexate on Retention of [^{3}H]Labeled DNA by BeWo Cells

	cpm Remaining		% Remaining
Time (hr)	Untreated	MTX-treated	MTX-treated
0	1830 ± 150		
24	1620 ± 140	1530 ± 80[1]	94.8 ± 7.9
48	1570 ± 190	1290 ± 50[1]	83.1 ± 8.7
72	n.d.	n.d.	
96	1280 ± 50	1170 ± 70[1]	91.4 ± 5.9
120	n.d.	n.d.	
144	1070 ± 60	320 ± 60[2]	30.3 ± 5.2

[1] *not significantly different from untreated (*$p>0.05$*)*

[2] *significantly different from untreated (*$p>0.05$*)*

BeWo cultures were established with 4 x 10^4 cells/20 ml glass scintillation vial, and after 24 hr, culture fluids were replaced with growth medium containing 2 nM [^{3}H]thymidine (0.1 μCi/flask, 50 Ci/mmol) as described in Table 3. After 24-hr incorporation intervals, culture fluids were replaced with drug-free medium (Untreated) or medium containing 1 μM methotrexate (MTX-treated). Culture fluids were replaced every 48 hr thereafter. Values represent the mean (±S.D., n=3) of radioactivity (cpm) associated with BeWo cells after the cultures were rinsed with 0.15 M NaCl and extracted 20 min at 4° with 0.2 M perchloric acid. "% Remaining MTX-treated" represents the amounts of radioactivity associated with drug-treated cultures expressed as a percentage of the amount remaining in drug-free cultures.

Table 4

Apparent Reuse of Prelabled DNA by BeWo cells

Culture	cpm Incorporated	Percent of Total[1]
Untreated	3710 ± 160	22.8 ± 1.1
MTX-treated	3210 ± 150	19.6 ± 1.1

[1] total radioactivity available from culture fluids was 18,640 cpm.

BeWo cultures were established with 4 x 10^4 cells/20 ml glass scintillation vial, and after 24 hr, culture fluids were replaced with 2 ml growth medium (with or without 1 μM methotrexate) which contained freeze-thawed BeWo cells prelabled with [^{3}H]thymidine (9230 cpm/ml, 10^5 cells/ml). BeWo cells were labeled with [^{3}H]thymidine and freeze-thawed as described in Materials and Methods (section A.4). After 48-hr exposures, cultures were rinsed with 0.15 M NaCl and extracted 20 min at 4° with 0.2 M perchloric acid, and the amount of radioactivity associated with cells (mean±S.D., n=5) was determined by liquid scintillation counting.

To determine if methotrexate induction of the CTL-to-STL differentiative response could be separated from methotrexate cytotoxicity, the viability of BeWo cells was assessed by vital staining and colony-forming ability. It is evident that the viability of cells subjected to 48-hr exposures to 1 μM methotrexate was unaffected (Table 5).

In the colony-forming assay used during this investigation, BeWo cells were subcultured at a density of 2 - 20 cells/cm^2 and were exposed to drug 24 hr after plating. If, at this extremely low population density, cells had not begun to proliferate 24 hr after subculturing, resistance to the cytotoxic effects of methotrexate could be related to the proliferative state of the cells. Quiescent or slowly proliferating cells are intrinsically resistant to the cytotoxic effects of methotrexate (15-17). An estimate of proliferation rates of untreated BeWo cells in the colony-forming assay was obtained by determing the number of cells per colony in methotrexate-free cultures (Table 6). There was a delay of 12 to 24 hr before cell numbers began to increase, and after 24 hr, cells actively proliferated, indicating that resistance to the toxic effects of methotrexate was not related to the proliferative status of the cells.

Table 5

Effects of 48-hr Exposures to 1 μM Methotrexate on the Apparent Viability of BeWo Cells

Method	Relative Viability
Trypan blue	101 ± 3 (5)[1]
Fluorescein diacetate	95 ± 5 (5)
Colony-forming ability	109 ± 11 (21)

[1] *number of separate determinations.*

BeWo cultures were established (2 x 10^5 cells/T-25 flask and 50 - 250 cells/60 mm tissue culture dish for vital staining and colony-forming assays, respectively), and after 24 hr, culture fluids were replaced with drug-free growth medium or medium containing 1 μM methotrexate, as described in Figure 5. After 48-hr exposures, viability was determined as described in Materials and Methods (section A.4). Values represent the apparent viability of methotrexate-treated cells (mean ± S.D.) and are expressed as a percentage of the viability of untreated cells determined by trypan blue (94 ± 3%, n=5), fluorescein diacetate (98 ± 2%, n=5), and colony-forming (67 ± 11%, n=24) methods.

Table 6

Effects of Low Population Density on BeWo Cell Proliferation

	Time (hr)				
Cells/Colony	12	24	48	72	96
1	79	67	27	20	24
2	8	28	17	8	1
3-4	8	6	15	17	8
5-8	5	2	19	26	25
9-16	0	1	7	13	19
17-32	0	0	13	12	12
33-64	0	0	3	4	8
65-128	0	0	0	0	2
Mean ± S.D.	1.5 ± 1.2	1.7 ± 1.5	9.0 ± 10.6	10.4 ± 9.9	15.8 ± 15.5

BeWo cultures were established with 250 cells/60 mm tissue culture dish (initial cell density of 10 cells/cm^2), and after 24 hr, culture fluids were replaced with drug-free growth medium (5 ml/culture) as described for the colony-forming assay (Materials and Methods, section A.4). Twelve hr after cultures were established, and at graded time intervals thereafter, cultures were stained with Wright's stain, and the number of cells/colony (100 colonies per culture) was counted. The mean number of cells/colony reported for 48 - 96 hr cultures did not include colonies with a single cell. The mean generation time (±S.D., 24 - 96 hr) of BeWo cultures, calculated from the mean number of cells/colony, was 16.6±6.5 hr.

2. Morphological differentiation

Morphological differentiation from the CTL to the STL phenotype during exposure to methotrexate has been well characterized (11). The morphology of proliferating and methotrexate-treated BeWo cells was examined in growth medium supplemented with undialyzed serum (Plate 1) and with dialyzed serum (Plate 2). Proliferating BeWo cultures were predominantly populated ($>$ 95%) with relatively small cells possessing a single nucleus (Plates 1A and 2A). Nucleoli were prominent and the number per nucleus ranged from 2 to 5. As cultures approached confluency, the cells became tightly associated and cell borders were indistinct. As observed previously (10, 11), also apparent at a low frequency ($<$ 5%) in proliferating BeWo cultures were giant cells that were often multinucleated. The nuclei of these cells were larger, in comparison to the nuclei of CTL cells, and were often multilobed. The giant cells are termed STL cells because of resemblance to syncytiotrophoblastic cells present in normal placenta (11). The appearance of STL cells in serially-transplanted BeWo cultures indicated that a small number of BeWo CTL cells possessed the capacity for spontaneous differentiation. During continous exposure to 1 μM methotrexate, cultures underwent a striking morphological differentiation to the STL phenotype (Plates 1B and 2B). The STL phenotype, characterized by increased cell size, nuclear enlargement, and multinucleation, was predominant 48-hr after exposures to 1 μM methotrexate were initiated. Comparison of the photomicrographs reproduced in plates 1 and 2 indicates that dialysis of the serum used to supplement the basal growth medium did not change the morphology of proliferating or methotrexate-treated BeWo cultures.

Plate 1. Morphological differentiation of BeWo cells during exposure to methotrexate: ***undialyzed serum*****.** BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with drug-free growth medium (A) or medium containing 1 μM methotrexate (B), as described in Figure 5. After 48-hr exposures, cultures were stained with Wright's stain for photomicroscopy. A spontaneously occuring STL cell is indicated by the arrow in the top micrograph, and a multinucleated STL cell induced by exposure to methotrexate is indicated by the arrow in the bottom micrograph. Magnification = 250x.

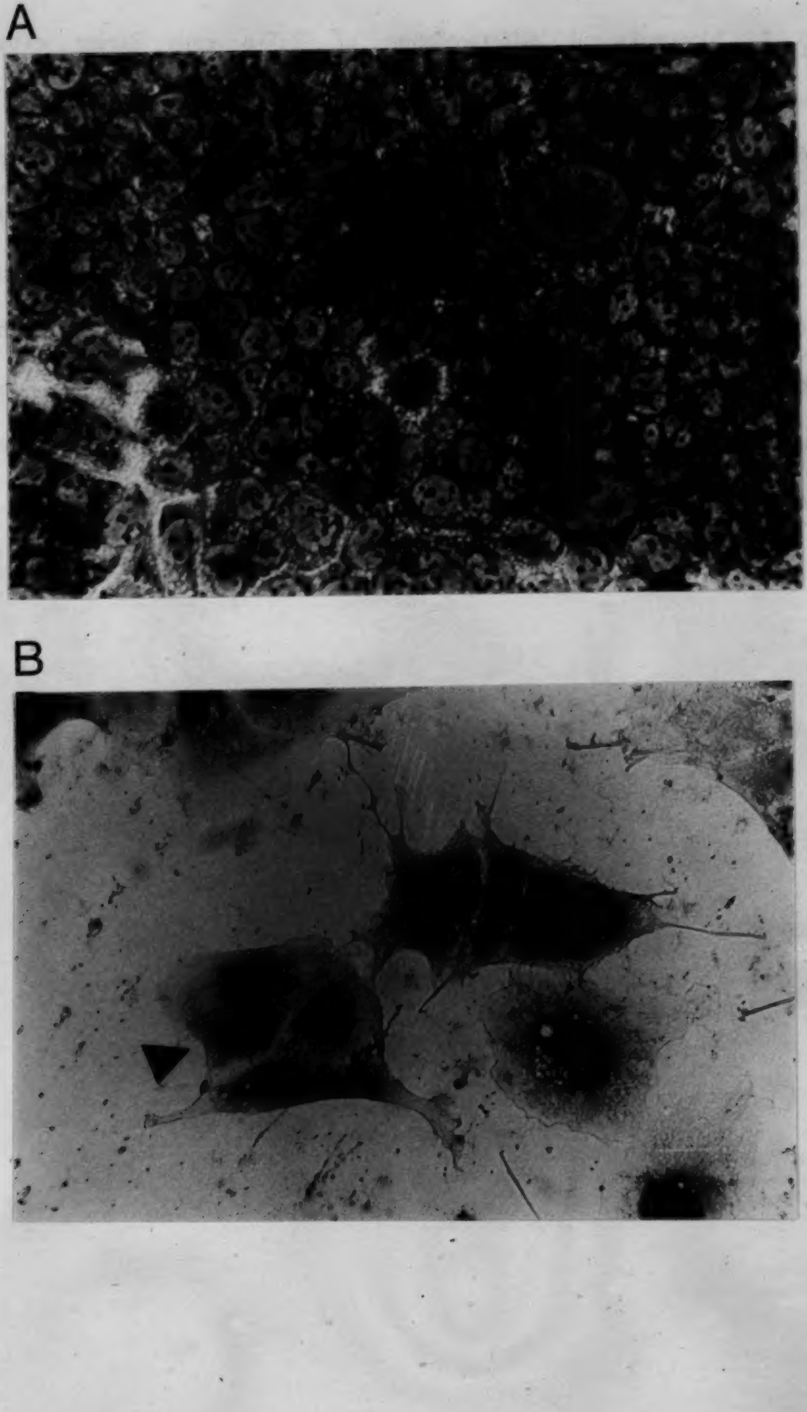
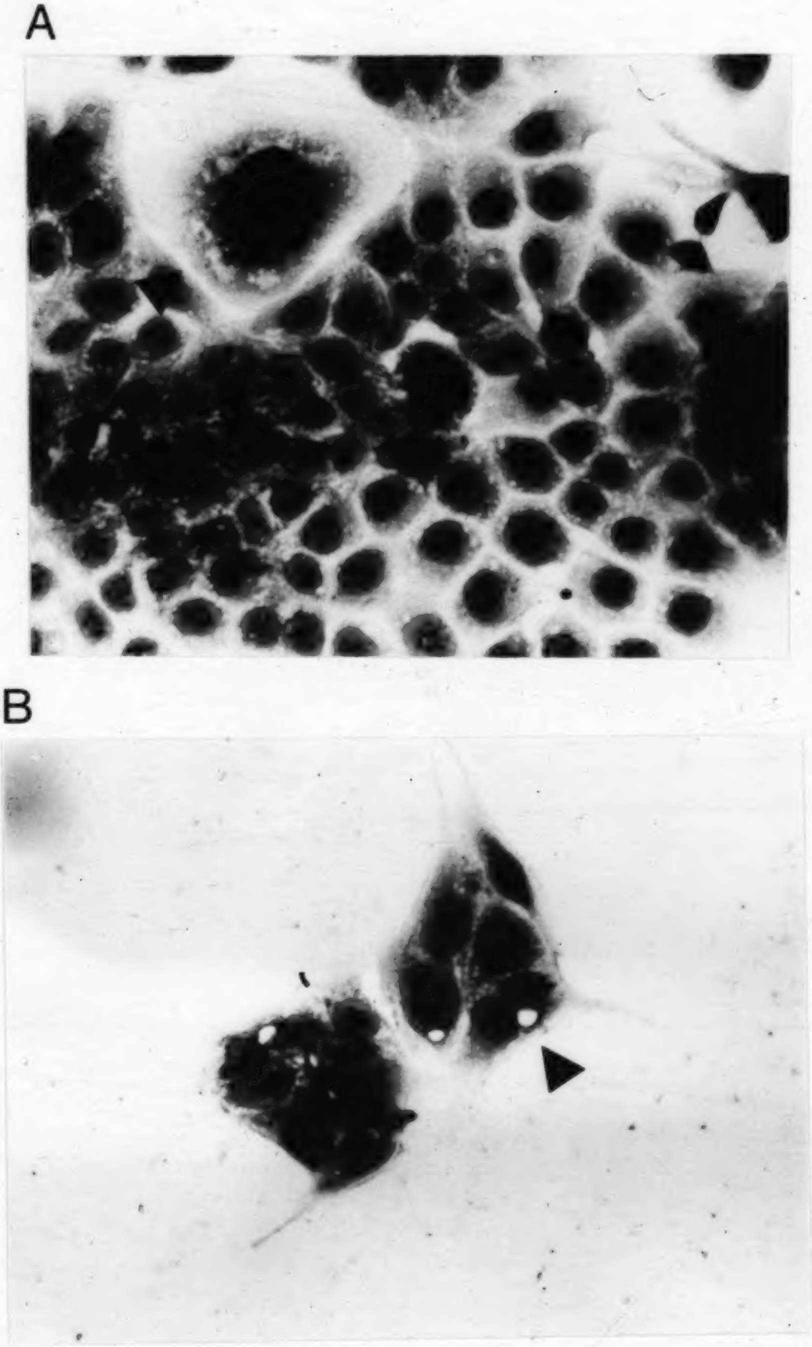

A
B

Plate 2. Morphological differentiation of BeWo cells during exposure to methotrexate: *dialyzed serum*. BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with drug-free growth medium (dFBS, A) or medium (dFBS) containing 1 μM methotrexate (B). After 48-hr exposures, cultures were stained with Wright's stain for photomicroscopy. A spontaneously occuring STL cell is indicated by the arrow in the top micrograph, and a multinucleated STL cell induced by exposure to methotrexate is indicated by the arrow in the bottom micrograph. Magnification = 250x.


A
B

3. Formation of giant cells

a. Methotrexate-induced increases in BeWo cell volume

Since morphological differentiation of BeWo cells to the STL phenotype was characterized by greatly enlarged cells, the effects of methotrexate on cell volume were quantitated electrically (Figure 7) and optically (Figure 8). It is evident that methotrexate induced considerable increases in cell volume. The largest cells present in cultures subjected to 48-hr exposures to methotrexate had volumes approximately 10 to 15-fold those of cells present in proliferating cultures. Population statistics for the volume distributions of Figure 7 are presented in Table 7. Since the median:mean ratios for volume distributions of proliferating and methotrexate-treated cells were virtually one, modal volume was the parameter used to report the effects of methotrexate on cell size.[4] Results of optical determinations of cell volume are shown in Figure 8.

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[4] Reporting modal volume afforded a significant technical advantage over mean or median volume. When upper or lower portions of volume distributions overlapped with debris or cell aggregates, analysis of the histogram was still possible. Furthermore, the orifice current and amplification settings of the Model Zf Coulter counter are discrete, rather than continous, and limit placement of the volume distribution within the range of the channel analyzer. It was impossible to set accurate upper and lower limits for relatively wide distributions (half-height width of 20-30 channels) with considerable debris or aggregates.

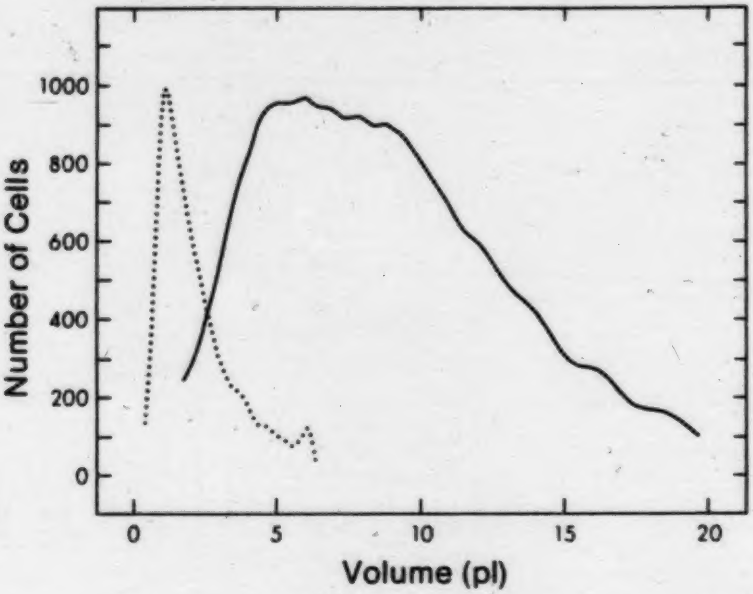


Figure 7. Effects of methotrexate on BeWo cell volume as determined electrically. BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with drug-free growth medium (···) or medium containing 1 μM methotrexate (—), as described in Figure 5. After 48-hr exposures (indicated by arrows in Figure 5), cell volumes were electrically determined as described in Materials and Methods (section B.1). The distributions presented are representative of many obtained under the experimental conditions of Figure 5.

Table 7

Population Statistics of BeWo Cell Volume Distributions

	Volume (pl/cell)	
Statistic	Untreated	MTX-treated
Mean ± S.D.	2.86 ± 1.38	10.94 ± 4.29
Median	2.63	10.62
Mode	1.57	9.43

Population statistics of the BeWo cell volume distributions presented in Figure 7 were calculated as described in Materials and Methods (section B.1). The average (±S.D.) median:mean ratios from a large series of experiments were, for untreated cells, 1.00±0.03 (n=37) and, for methotrexate-treated cells (48-hr exposures to 1 μM methotrexate), 0.98±0.04 (n=22).

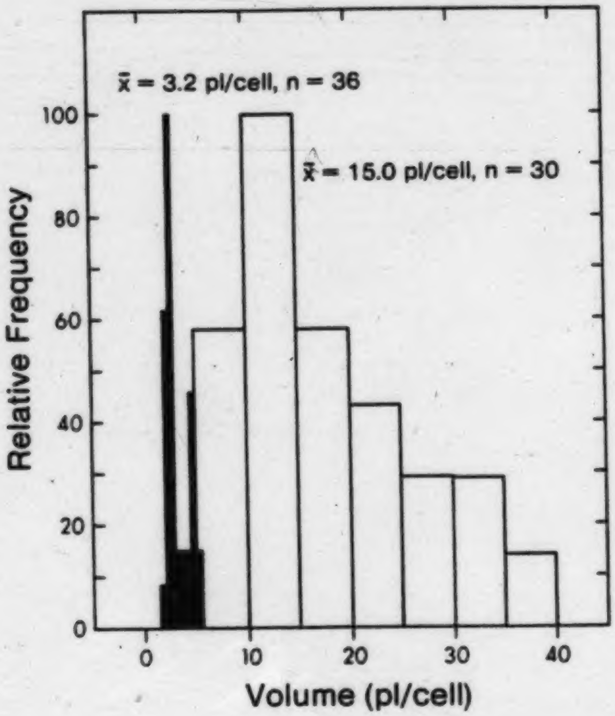


Figure 8. Effects of methotrexate on BeWo cell volume as determined optically. BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with drug-free growth medium (solid bars) or medium containing 1 μM methotrexate (open bars), as described in Figure 5. After 48-hr exposures, cells were disaggregated with trypsin-EDTA, suspended in 0.15 M NaCl, and wet-mounts were prepared. Major and minor diameters of proliferating and methotrexate-treated BeWo cells were determined from photomicrographs, averaged, and used to calculate volumes assuming that cells were spherical.

b. RNA and protein content

The effects of methotrexate on RNA and protein content of BeWo cultures were assessed to determine if increased cell volume observed in the experiments of Figures 7 and 8 represented increased cellular RNA and protein. Cultures were exposed to methotrexate under conditions that completely inhibited BeWo cell proliferation (see Figure 5); thus, any increase in RNA or protein content of such cultures represented increased RNA and protein content per cell. The RNA content of drug-treated cultures did not significantly increase after the first 24 hr of exposure to 1 μM methotrexate, whereas the protein content of such cultures was still increasing after 72-hr exposure to drug (Table 8). Similar increases in protein content during continous exposure to 1 μM methotrexate have been previously reported (11, 13).

3. Effects of serum on giant cell formation

To determine the effects of serum components on methotrexate-induced increases in cell mass, the volumes of untreated and methotrexate-treated BeWo cells were determined at 24-hr intervals during 5-day exposures to methotrexate in growth medium supplemented with dialyzed or undialyzed serum (Figure 9). Although the volume of proliferating cells was the same when cells were maintained in either sera, the volume of drug-treated cells was significantly less when cultures were maintained in dialyzed than in undialyzed serum. These differences suggested that methotrexate-induced increases in cell volume required a dialyzable factor(s) present in serum. The factor(s) was not required for growth of BeWo cells since populating doubling times of cultures maintained in undialyzed and dialyzed sera were the same (section B.1).

The averages (±S.D.) of a large series of volume determinations obtained after 48-hr exposures to methotrexate in serum-free, dialyzed, and undialyzed sera are presented in Table 9. It is evident that methotrexate-induced increases in cell volume required both dialyzable and undialyzable serum factors. The dialyzable factor was subsequently identified as hypoxanthine (Chapter VI). The undialyzable factor(s) was not identified.

Table 8

RNA and Protein Content of Methotrexate-treated BeWo Cultures

Time (hr)	RNA (μg)	Protein (μg)
0	54.4 ± 2.0	110 ± 2
24	78.2 ± 5.6	164 ± 3
48	78.4 ± 8.3	195 ± 3
72	79.9 ± 6.6	215 ± 4

BeWo cultures were established with 2 x 10^5 cells/2-oz prescription bottle, and after 24 hr, culture fluids were replaced with growth medium containing 1 μM methotrexate as described in Figure 5. The amounts (mean ± S.D.) of RNA (n=6) and protein (n=3) per flask were determined at 24-hr intervals as described in Materials and Methods (section E.2).

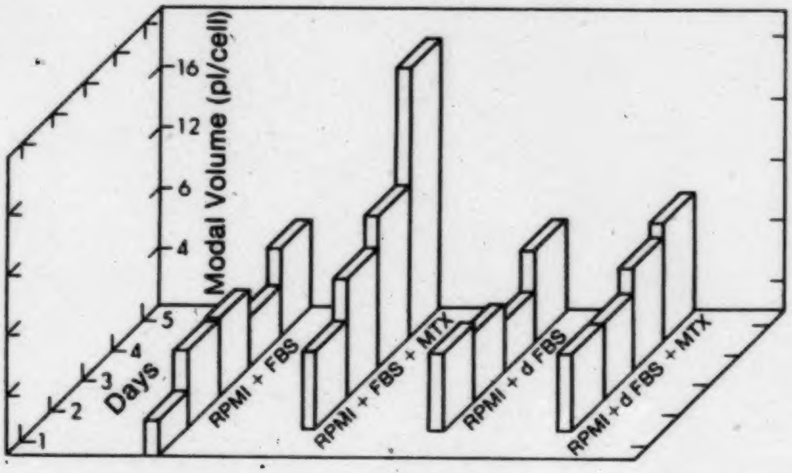


Figure 9. Effects of methotrexate on the volume of BeWo cells cultured in growth medium supplemented with dialyzed or undialyzed fetal bovine serum. BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 and 72 hr, culture fluids were replaced with drug-free medium or medium containing 1 μM methotrexate supplemented with undialyzed (RPMI + FBS) or dialyzed serum (RPMI + dFBS), as described in Figure 5. Each bar represents the modal cell volume (average, n = 3) determined electrically as described in Materials and Methods (section B.1).

Table 9

Effects of Serum on Methotrexate-induced Increases in Cell Volume

	Modal Volume (pl/cell)	
Serum Supplement	Untreated	MTX-treated
FBS	3.51 ± 1.29 (20)[1]	10.28 ± 1.27 (9)
dialyzed FBS	3.34 ± 0.72 (27)	6.49 ± 1.47 (13)
serum-free	2.38 ± 0.17 (9)	3.92 ± 0.39 (9)

[1] *number of separate determinations*

BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with drug-free medium or medium containing 1 μM methotrexate and the appropriate serum supplement, as described in Figure 8. After 48-hr exposures, volumes (average±S.D.) were electrically determined as described in Materials and Methods (section B.1).

4. Alkaline phosphatase activity

Human placenta expresses three distinct isoenzymes of alkaline phosphatase at various developmental stages. During the first 10 weeks of gestation, two isoenzymes are expressed that are immunologically dissimilar to term placental alkaline phosphatase. One of the two isoenzymes present in the first weeks of gestation is apparently specific to the placenta since it was not immunoprecipitated by antisera against adult liver, intestinal, or bone alkaline phosphatase (22). The other isoenzyme is indistinguishable from liver alkaline phosphatase (22). After 14 to 16 weeks of gestation, term alkaline phosphatase is expressed by the placenta, and developmentally earlier forms cannot be detected (22, 23). Alkaline phosphatase activity of term placenta is stable at 60°, whereas liver, bone, intestinal, and the early placental isoenzymes are heat-labile (22, 24).

1. Histochemical localization

Expression of alkaline phosphatase by cultured BeWo cells has been used as a biochemical marker of syncytiotrophoblast (1). The specific activity of alkaline phosphatase in crude extracts of proliferating BeWo cultures is relatively low in comparison to that of methotrexate-treated cultures (1). Alkaline phosphatase was histochemically localized in morphologically identifiable STL cells in untreated and methotrexate-treated cultures (Table 10). In untreated cultures, the majority of spontanously occuring STL cells expressed relatively high levels of alkaline phosphatase, whereas activity was evident in the cytoplasm of CTL cells only after prolonged exposures to the chromogenic substrate (1-2 hr, data not shown). These results suggested that spontaneously occuring STL cells were responsible for most of the alkaline phosphatase activity previously reported in extracts of proliferating BeWo cultures (1).

Expression of alkaline phosphatase activity in methotrexate-treated BeWo cultures was not uniform. In the experiment of Table 10 approximately half the STL cells in methotrexate-treated cultures were strongly positive for alkaline phosphatase, whereas the remainder were weakly positive (data not shown), staining only after prolonged exposure to the substrate (> 1 hr).

2. Enzymatic assay for alkaline phosphatase.

The placental isoenzyme of alkaline phosphatase can be identified by characterization of heat-stability and pH optima (22, 24). The effects of heating and pH on alkaline phosphatase activity in extracts of proliferating BeWo cells and serum obtained from a pregnant woman were compared in the experiments of Figure 10. Alkaline phosphatase from term placenta can be detected in the serum of pregnant women (25). Approximately 80% and 10% of the alkaline phosphatase activity in extracts of BeWo cells and in the serum of a pregnant woman, respectively, was stable at 60°, and the heat-stable forms of the enzyme expressed in BeWo cell extracts and in serum exhibited similar pH activity-profiles with a optima of pH 10.

Thus, approximately 80% of the total alkaline phosphatase activity expressed by proliferating BeWo cells was indistinguishable from alkaline phosphatase in serum during pregnancy. Similar values for the relative amount of term-placental isoenzyme expressed by BeWo cells, determined enzymatically (70%) and immunochemically (76%), have been previously reported (26, 27).

Before using alkaline phosphatase as a biochemical marker of the CTL-to-STL differentiative response, the effect of methotrexate on the assay for the enzyme was determined (Table 11). The activity of heat-stable alkaline phosphatase was unaffected by the presence of methotrexate at concentrations comparable to those used in culture fluids during drug exposures. Thus, changes in the activity of heat-stable alkaline phosphatase in extracts of BeWo cells after exposure to methotrexate were not due to effects of methotrexate on the assay itself.

Table 11

Histochemical Localization of Alkaline Phosphatase Activity

Cell Type	Relative Frequency	Percent Positive
Spontaneous STL	< 5	64 ± 6
MTX-induced STL	80 - 90	58 ± 6

BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with drug-free growth medium (spontaneous STL cells) or growth medium containing 1 μM methotrexate (MTX-induced STL cells). After 48-hr exposures, cultures were incubated 15 min with a chromogenic substrate for alkaline phosphatase as described in Materials and Methods (section G.1). Values represent the number (mean ± S.D.) of morphologically identified and alkaline phosphatase positive STL cells determined by scoring 100-500 cells/culture (3 cultures for each condition).

Figure 10. A: Heat-stability of alkaline phosphatase in extracts of BeWo cells and serum from a pregnant woman. BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with drug-free growth medium. After 48 hr, extracts were made and pooled (Materials and Methods, section G). Serum from a pregnant woman was obtained as described in Materials and Methods (section G). After heating cell-extracts or serum samples in a 60° water-bath for the time intervals indicated, portions were removed, cooled to 4°, and the activity of alkaline phosphatase was measured by hydrolysis of *p*-nitrophenyl phosphate (Materials and Methods, section G). **B: pH optima of the heat-stable isoenzyme of alkaline phosphatase present in extracts of BeWo cells and serum from a pregnant woman (week 26 of gestation).** Extracts of BeWo cells or serum, obtained as described above, were heated 5 min at 60° and the apparent activities of alkaline phosphatase were determined by hydrolysis of *p*-nitrophenyl phosphate at the indicated pH. Buffers used to determine the pH optimum of heat-stable alkaline phosphatase were: pH 3-6; 0.2 M succinate, pH 6-9; 0.2 M hepes, pH 9-12; 0.2 M 2-amino-2-methyl-1-propanol.

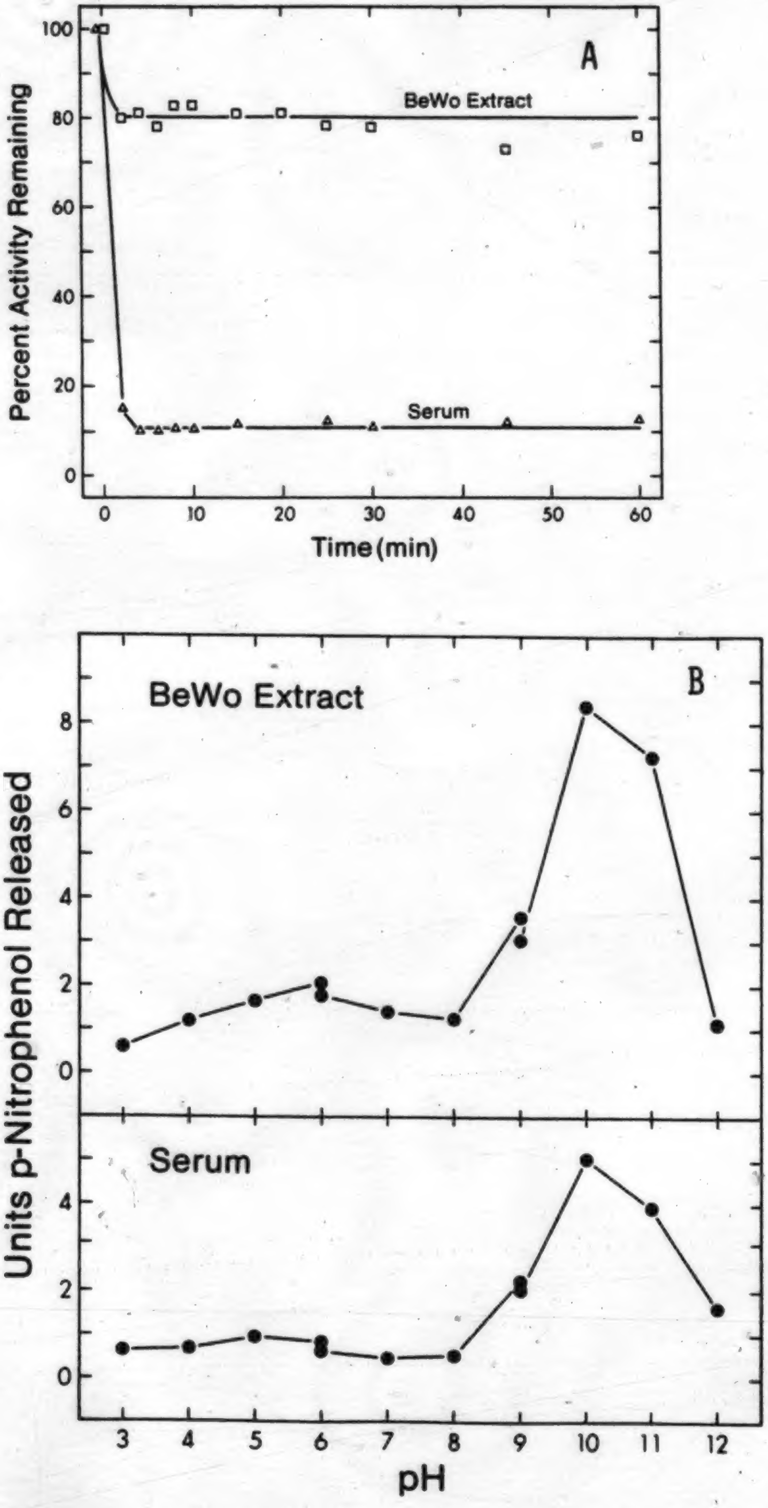

A
Percent Activity Remaining
100
80
60
40
20
0
BeWo Extract
Serum
0
10
20
30
40
50
60
Time (min)
B
Units p-Nitrophenol Released
BeWo Extract
8
6
4
2
0
Serum
4
2
0
3
4
5
6
7
8
9
10
11
12
pH

Table 11

Effects of Methotrexate on the Activity of Heat-stable Alkaline Phosphatase

Methotrexate (μM)	Specific Activity (nmol/min/mg protein)
0.0	18.5 ± 6.3
0.29	16.4 ± 5.5
2.90	17.0 ± 6.1
29.0	16.0 ± 6.3

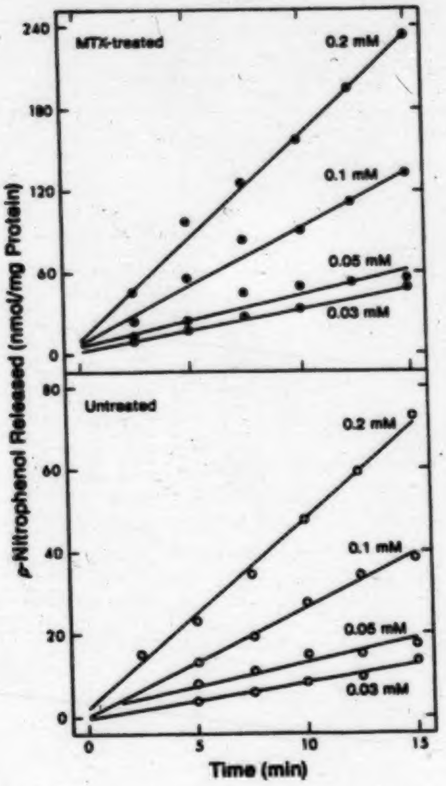
BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with drug-free growth medium. After 48 hr, extracts of cultures were made and heated 5 min at 60° as described in Materials and Methods (section G). The specific activity (mean±S.D., n=3 separate cultures assayed in triplicate) of heat-stable alkaline phosphatase was determined by hydrolysis of *p*-nitrophenyl phosphate. The reaction mixtures consisted of 100 μl of a crude-extract of BeWo cells, 100 μl of water with or without methotrexate, and 500 μl of an 8 mM solution of *p*-nitrophenyl phosphate in 0.5 M 2-amino-2-methyl-1-propanol (pH 10). Reagent blanks were the same except that 100 μl water was substituted for the BeWo cell extract.

c. Kinetics of heat-stable alkaline phosphatase

A kinetic study was undertaken with heat-stable alkaline phosphatase to further characterize the isoenzyme expressed by proliferating and methotrexate-treated It is apparent from the data presented in Figure 11 that the rate of hydrolysis of *p*-nitrophenyl phosphate was linear with respect to time for at least 15 min over the substrate concentration range of 0.03 to 0.2 mM. In crude extracts of proliferating and methotrexate-treated BeWo cells, the Km values, respectively, for *p*-nitrophenyl phosphate were 0.93 and 1.00 mM and the Vmax values were 28.9 and 87.7 nmol/min/mg protein. In a parallel experiment, a value of 1.48 mM was obtained with commercially obtained alkaline phosphatase from term placenta. Thus, although the apparent activity of heat-stable alkaline phosphatase was increased 3-fold the affinity of the enzyme for *p*-nitrophenyl phosphate was not altered. This value was similar to previously reported values for the affinity of placental alkaline phosphatase from term placenta for phenyl phosphate derivatives (Table 12). Thus, these results indicate that the predominant isoenzyme of alkaline phosphatase of BeWo cells was indistinguishable from placental alkaline phosphatase of term placenta on the basis of heat-stability, pH optima, and affinity for *p*-nitrophenyl phosphate.

The rate of hydrolysis of 5.7 mM *p*-nitrophenyl phosphate in BeWo cell extracts was linearly related to the amount of enzyme present in reaction mixtures (Figure 12). Heat-stable alkaline phosphatase was saturated under the conditions used for assay of activity in crude extracts, and the specific activities determined in subsequent sections as described in Materials and Methods (section G) represent Vmax/mg protein.

Figure 11. Effects of 1 μM methotrexate on the Km and Vmax of heat-stable alkaline phosphatase expressed by BeWo cells. A: BeWo cultures were established with 1 x 10^6 cells/T-75 flask. and after 24 hr, culture fluids were replaced with drug-free growth medium or medium containing 1 μM methotrexate. After 48-hr exposures, extracts of proliferating and methotrexate-treated BeWo cultures were made, and after heating the extracts at 60° for 5 min, the apparent activity of heat-stable alkaline phosphatase was determined by hydrolysis of *p*-nitrophenyl phosphate as described in Materials and Methods (section G). B: Initial velocities were determined from the time courses in Panel A by linear regression and the reciprocals were plotted as a function of *p*-nitrophenyl phosphate (Lineweaver-Burke plot).


MTX-treated
0.2 mM
0.1 mM
0.05 mM
0.03 mM
Untreated
0.2 mM
0.1 mM
0.05 mM
0.03 mM
p-Nitrophenol Released (nmol/mg Protein)
240
180
120
60
0
80
60
40
20
0
0
5
10
15
Time (min)

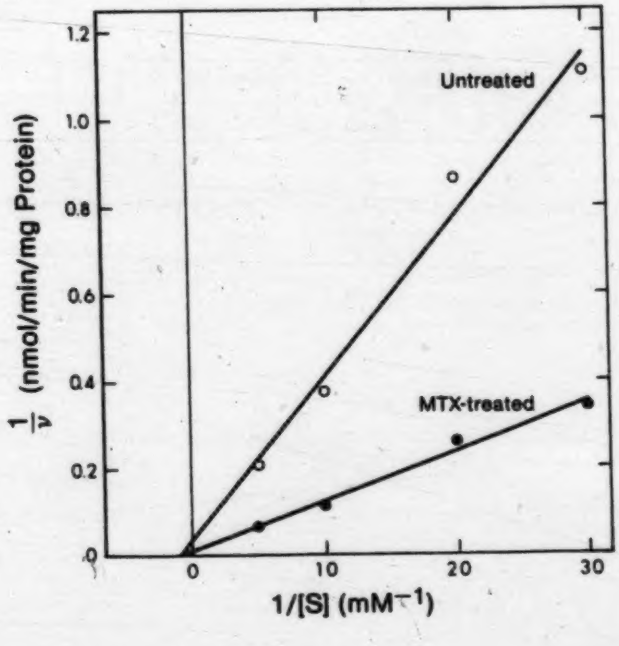

Untreated
MTX-treated
$\frac{1}{v}$ (nmol/min/mg Protein)
1.2
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
1/[S] (mM^{-1})

Table 12

Phenyl Phosphate Derivatives as Substrates

for Alkaline Phosphatase from Term Placenta

Phosphate Derivative	Km (mM)	Reference
p-nitrophenyl	0.8	28
p-nitrophenyl	1.48	this study
phenyl	1.89	12
phenyl	1.20	29

The velocity of commercially obtained alkaline phosphatase from term placenta at pH 10 was determined from the amount of *p*-nitrophenyl phosphate released during a 15-min incubation period at 37° (Materials and Methods, section G). The substrate concentrations ranged from 0.0125 to 5 mM, and the coefficient of determination (r^2) from least-squares linear regression analysis of the Lineweaver-Burke plot was 1.00 (n=12). The Vmax of placental alkaline phosphatase was 15.6 μmol *p*-nitrophenol released/min/mg protein, and 9 μg protein was used per assay.

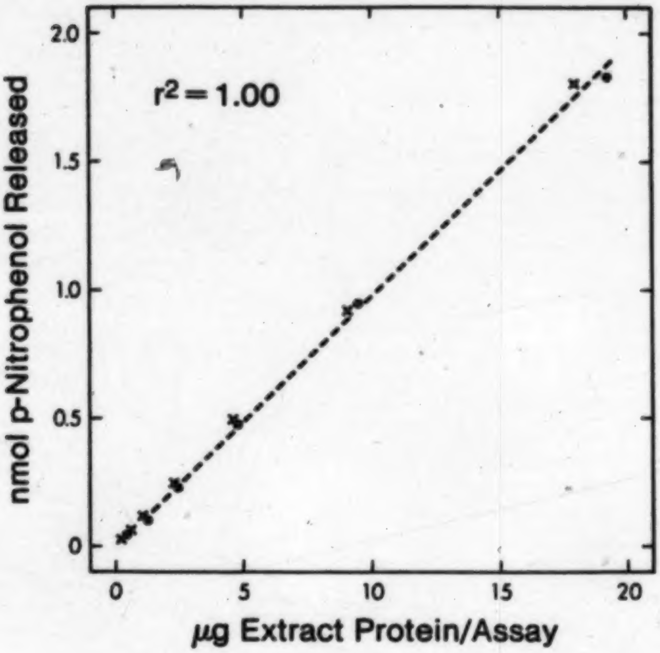


Figure 12. Effects of enzyme concentration on the rate of hydrolysis of *p*-nitrophenyl phosphate at pH 10. BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with drug-free growth medium, as described in Figure 5. After 48 hr, heat-stable alkaline phosphatase was assayed by hydrolysis of *p*-nitrophenyl phosphate (5.7 mM) as described in Materials and Methods (section G). Each point represents the rate of hydrolysis at a different enzyme concentration prepared by varying the amount of cell extract (two separate extracts indicated by different symbols) in the assay. The dashed line and the coefficient of determination (r^2) were determined by linear regression using the method of least squares.

d. Effects of methotrexate on expression of heat-stable alkaline phosphatase

The effects of methotrexate on the specific activity of the heat-stable isoenzyme of alkaline phosphatase expressed by BeWo cells was assessed to determine the extent to which expression of this biochemical marker of *in utero* placental trophoblast was altered during methotrexate-induced differentiation to the STL phenotype. A 3-fold increase in the specific activity of heat-stable alkaline phosphatase was evident after 96 hr exposures to 1 μM methotrexate (Table 13). In contrast, the specific activity of alkaline phosphatase was relatively stable in proliferating BeWo cultures over the time course of the experiment. It has been previously shown that a 24-hr pulsed exposure to methotrexate resulted in a transitory 3-fold increase, that persisted for 2 d, in the specific activity of the heat-stable form of alkaline phosphatase expressed by BeWo cells (1).

In section 3.c it was demonstrated that maximal increases in BeWo cell volume during exposure to methotrexate required dialyzable and undialyzable serum factors. In the experiment of Table 14, the effect of dialysis of serum on expression of heat-stable alkaline phosphatase by BeWo cells was determined. In both untreated and methotrexate-treated cells, the activity of heat-stable alkaline phosphatase was unaffected by dialysis of serum, indicating that methotrexate-stimulation of alkaline phosphatase expression did not require exogenous factors.

Table 13

Effects of Methotrexate on Expression of Heat-stable Alkaline Phosphatase by BeWo Cells

	Specific Activity (nmol/min/mg protein)	
Time (hr)	Untreated	MTX-treated
0	19.5 ± 2.1	
24	22.6 ± 10.3	22.3 ± 1.1
48	13.6 ± 2.2	32.0 ± 3.4
72	23.6 ± 0.8	51.1 ± 3.1
96	24.3 ± 1.5	76.0 ± 17.5

BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with drug-free growth medium or medium containing 1 μM methotrexate. At 24-hr intervals, extracts of proliferating (Untreated) and 1 μM methotrexate-treated (MTX-treated) cultures were made and heated at 60° for 5 min, and the specific activity (mean±S.D., n=3 separate cultures assayed in triplicate) of alkaline phosphatase was determined as described in Materials and Methods (section G).

Table 14

Effects of Dialysis of Serum on Expression of Heat-stable Alkaline Phosphatase by BeWo Cells

	Specific Activity (nmol/min/mg protein)	
Supplement	Untreated	MTX-treated
FBS	14.6 ± 2.7	57.3 ± 22.9
Dialyzed FBS	12.9 ± 3.1[1]	76.6 ± 14.1[2]

[1] *not significantly different from untreated in the presence of FBS* ($p>0.05$).

[2] *not significantly different from MTX-treated in the presence of FBS* ($p>0.05$).

BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24-hr, culture fluids were replaced with drug-free growth medium or medium containing 1 μM methotrexate supplemented with either 10% dialyzed or undialyzed fetal bovine serum (FBS). After 48-hr exposures, extracts of proliferating (Untreated) or methotrexate-treated (MTX-treated) cultures were made, and heat-stable alkaline phosphatase activity (mean ± S.D., n=9 separate cultures assayed in triplicate) was determined as described in Materials and Methods (section G).

5. Concentration-effect relationships

The relationships between methotrexate and its effects on viability, proliferation rates, and two markers of the CTL-to-STL differentiative response were assessed in the experiments of Figure 13. Inhibition of cell proliferation, formation of giant cells, and differentiation to the STL phenotype exhibited similar concentration-effect relationships, suggesting that methotrexate action at a single biochemical target resulted in both the antiproliferative and cytodifferentiative effects of the drug. In contrast, colony forming ability of BeWo cells was not decreased over the concentration range tested. Thus, although BeWo cells were sensitive to the antiproliferative effects of methotrexate, reproductive capacity was not decreased after exposures to methotrexate that completely inhibited cell proliferation.

Colonies arising after exposure to methotrexate in the viability assay consisted of 50 to several hundred cells that were predominantly (> 90%) CTL in phenotype, suggesting that, when placed in drug-free medium, BeWo cells reverted to the CTL phenotype. Six colonies were expanded after 48-hr exposures to 1 μM methotrexate and all cells of these clonal lines displayed the CTL phenotype (Plate 3A). The effects of methotrexate on the morphology of one of these lines was assessed to determine if cells derived from colonies that formed after 48-hr exposures to methotrexate had the capacity to differentiate to the STL phenotype (Plate 3B). About 90% of cells present in BeWo/MT1 cultures exposed to methotrexate for 48 hr acquired the STL phenotype suggesting that the cells from which these clones were derived were not intrinsically resistant to the cytodifferentiative effects of methotrexate.

6. Toxic effects of methotrexate in other cell types

All of the existing choriocarcinoma cell lines were derived from tumors obtained from patients that had become clinically resistant to methotrexate chemotherapy. There are no examples of cell lines that were derived from patients who were sensitive to methotrexate therapy. The sensitivity of other cell types to the cytotoxic effects of methotrexate were determined under culture conditions similar to those used for experiments with BeWo cells. It

Figure 13. Methotrexate concentration-effect relationships for reproductive viability, proliferation, formation of giant cells, and differentiation to the STL phenotype. BeWo cultures were established (50-500 cells/60 mm tissue culture dish for viability determinations; 2×10^5 cells/T-25 flask for determination of drug effects on proliferation, cell volume, and morphology) in drug-free growth medium, and after 24 hr, culture fluids were replaced with drug-free medium or medium containing the concentration of methotrexate indicated. After 48-hr exposures, colony-forming ability, cell volume, morphology, and number of doublings were determined. Reproductive capacity was assessed by colony-forming ability (Materials and Methods, section A.4) and values represent viability of drug-treated cells as a percentage of that of proliferating BeWo cells (66.8±10.5%, n=24). The number of doublings were calculated from cell numbers determined 24 and 72 hr after cultures were initiated. Modal cell volume (average±S.D, n=3) was determined electrically as described in Materials and Methods (section B.1). Values for the percentage of cells displaying the CTL or STL phenotype were obtained from scoring 100 cells/culture (n=1) at the concentration of methotrexate indicated.

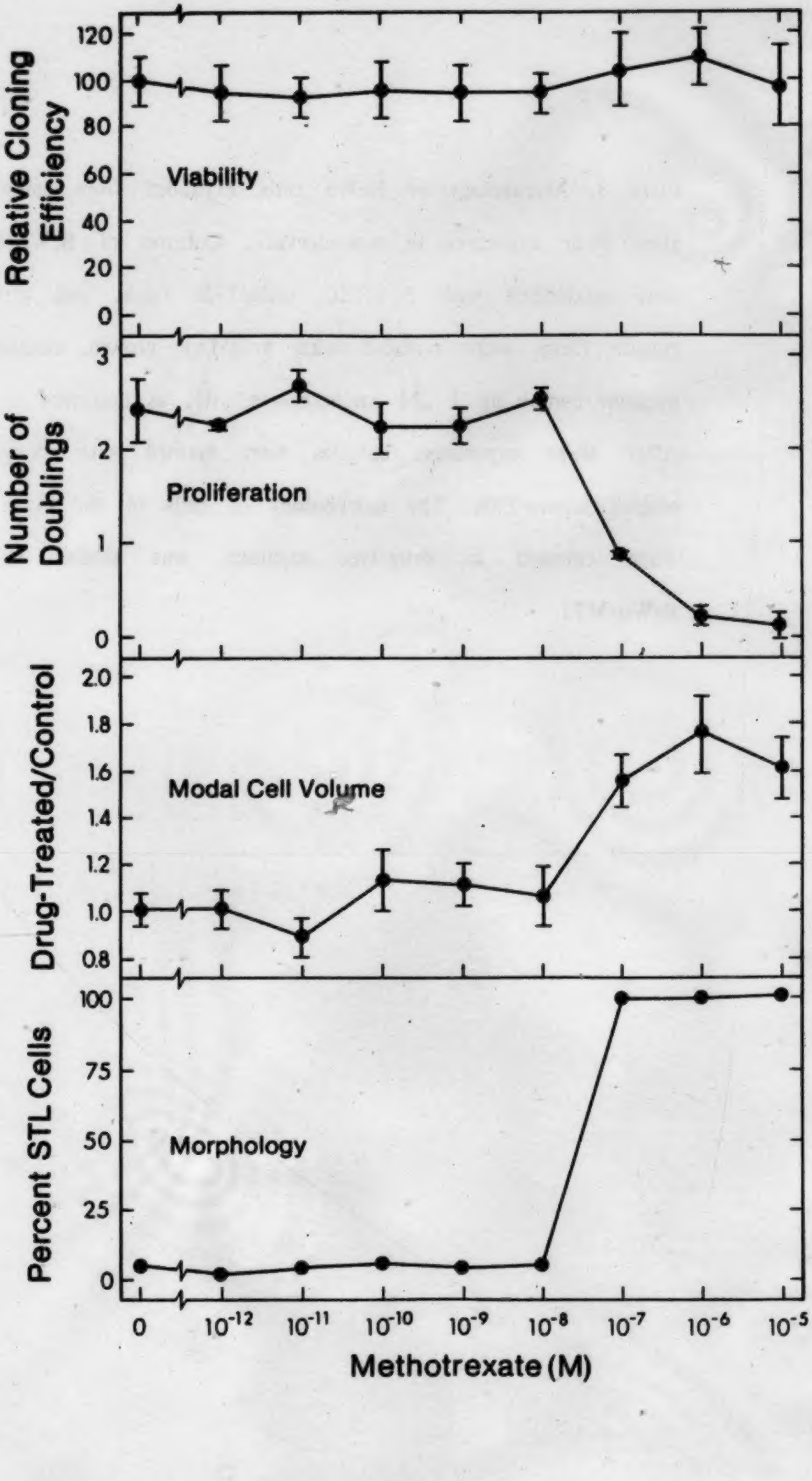
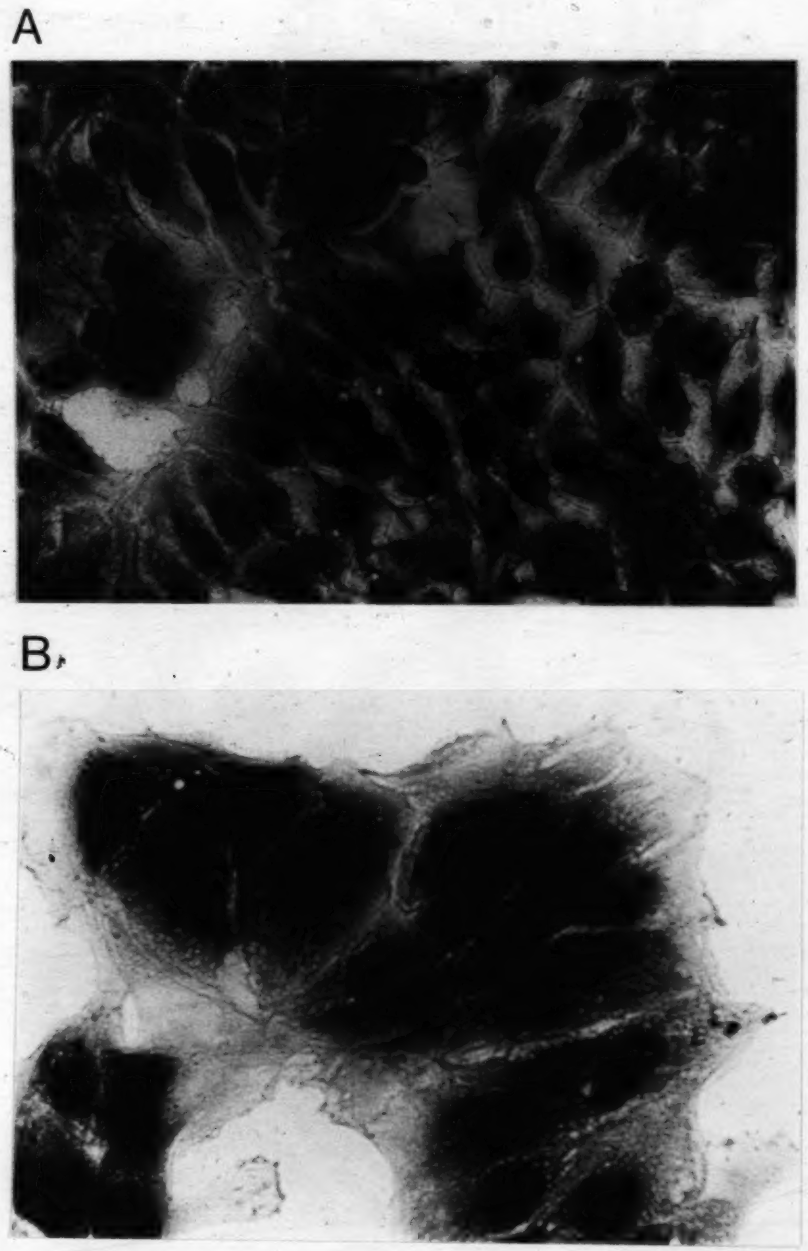

Relative Cloning Efficiency
120
100
80
60
40
20
0
Viability
Number of Doublings
3
2
1
0
Proliferation
Drug-Treated/Control
2.0
1.8
1.6
1.4
1.2
1.0
0.8
Modal Cell Volume
Percent STL Cells
100
75
50
25
0
Morphology
0
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Methotrexate (M)

Plate 3. Morphology of BeWo cells expanded from colonies arising after 48-hr exposures to methotrexate. Cultures of BeWo/MT-1 cells were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with drug-free growth medium (A) or medium containing 1 μM methotrexate (B), as described in Figure 5. After 48-hr exposures, cultures were stained with Wright's stain. Magnification=250x. The morphology of cells of the other 5 clones, when cultured in drug-free medium, was similar to that of BeWo/MT1.


A
B

is evident from data presented in Table 15 that BeWo cells were relatively resistant to the cytotoxic effects of methotrexate, but HeLa cells also displayed resistance to the toxic effects of the drug under the conditions of this experiment.

BeWo, HeLa, HL-60, and 3924A cultures were established as described in Materials and Methods (section A), and after 24 hr, culture fluids were replaced with drug-free growth medium or medium containing 1 μM methotrexate. After 48-hr exposures, viability of cultured cells was determined by colony-forming assays as described in Materials and Methods (section A). Viability of drug-treated cultures was expressed as a percentage of the colony-forming ability of proliferating cultures of BeWo cells (67±11, n=24/3) HeLa (40±9, n=20/1), HL-60 (33±6, n=19/1), and 3924A (55±5, n=20/1). Literature values (30) for the effects of methotrexate on cell viability were determined in colony-forming assays under the same conditions of this study, and viability was expressed as a percentage of the viability of untreated cells as determined from graphs. Values for the apparent viability of proliferating cultures are for PMC-22 (85±13), L1210 (100±13), and CCRF-CEM (100).

Table 15

Cytotoxic Effects of Methotrexate

Cell Type	MTX, µM	Relative Viability	Reference
BeWo	1	109 ± 11 (21/3)[1]	this study
	10	98 ± 17 (13/3)	"
HeLa	10	97 ± 30 (21/3)	"
HL-60	10	< 1.5 (20/1)	"
Morris Hepatoma (3924A)	10	2.0 ± 1.3 (29/1)	"
Human Melanoma PMC-22	1	8	30
Mouse Leukemia L1210	1	3	30
Human T-cell Leukemia CCRF-CEM	0.1	22 ± 5	30
	1	22	30
	10	11	30

[1] *number of separate cultures/ number of experiments*

C. Summary

The work described in this Chapter was undertaken to characterize expression of syncytiotrophoblastic markers by BeWo cells and to determine the relationships between differentiation and the antiproliferative and cytotoxic effects of methotrexate. Although the BeWo cell line was derived from a malignant choriocarcinoma obtained from a patient that became refractory to methotrexate chemotherapy, BeWo cells were exceedingly sensitive to the antiproliferative effects of methotrexate. After intervals of exposure to methotrexate that were were lethal to most other cell types, the reproductive capacity of BeWo cells was unaffected. Methotrexate-treated BeWo cells underwent a cytodifferentiative response that was a caricature of normal syncytiotrophoblastic development, and only after prolonged exposures to 1 μM methotrexate was there significant loss of viability. These observations indicate that although BeWo cells were highly sensitive to the cytostatic effects of methotrexate, they were relatively resistant to the cytotoxic effects of the drug. The reversible formation of nonproliferative STL cells during exposure to methotrexate may represent a novel biological mechanism of resistance.

Under culture conditions permissive for the CTL-to-STL differentiative response, BeWo cells coordinately expressed increases in volume, elevated levels of alkaline phosphatase, and morphological differentiation to the STL phenotype. Increases in cell volume correlated with increases in both RNA and protein content per culture, and the predominant form of alkaline phosphatase expressed was indistinguishable from that of term placenta on the basis of heat-stability, pH optima, and affinity for *p*-nitrophenyl phosphate. It was previously shown that approximately 80% of BeWo cell alkaline phosphatase was immunoprecipitated by antibodies against alkaline phosphatase of term placenta (27). Thus, enzymatic identification of the isoenzyme expressed by BeWo cells was consistent with immunological identification. Similar concentration-effect relationships for expression of syncytiotrophoblastic markers and inhibition of proliferation by methotrexate suggest that methotrexate-action at a single biochemical target was responsible for both effects.

During these investigations, it became apparent that the serum used to supplement the basal growth medium altered methotrexate-induced formation of giant cells. It was demonstrated that methotrexate-induced increases in cell volume, but not expression of morphological changes or stimulation of alkaline phosphatase activity, were less in cultures maintained in growth medium supplemented with dialyzed fetal bovine serum. Experiments undertaken to identify the dialyzable factor required for giant cell formation are reported in Chapter VI.

D. References

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VI. Identification of Hypoxanthine as a Factor Required for Giant Cell Formation

A. Introduction

The experiments described in Chapter V established that methotrexate-induced giant cell formation, but not expression of heat-stable alkaline phosphatase or morphological differentiation to the STL phenotype, required a dialyzable serum factor(s). Experiments presented in this chapter were undertaken to identify the dialyzable factor(s) necessary for methotrexate-induced formation of giant BeWo cells.

Increases in BeWo cell volume during exposure to methotrexate were accompanied with increases of cellular RNA (Chapter V). If methotrexate acts through inhibition of dihydrofolate reductase in BeWo cells, then it is unlikely that *de novo* purine synthesis would be sufficient to support production of RNA (1-5). Hence, the possibility that a salvagable purine was the dialyzable serum factor required for methotrexate-induced increases in cell volume was investigated. Increases in both RNA and protein content of BeWo cells were less when cultures were exposed to methotrexate in growth medium supplemented with dialyzed serum in comparison to undialyzed serum, and increases in RNA content were inhibited more than increases in protein suggesting that exogenous purines were required for continued nucleic acid synthesis. The effects of specific methods for removing purines from sera (charcoal-extraction or xanthine oxidase-treatment) on methotrexate-induced increases in cell volume were assessed. The results of these experiments indicated that either xanthine or hypoxanthine was the dialyzable factor. The purine content of fetal bovine serum was measured, and the ability of authentic hypoxanthine and other purines to augment methotrexate-induced increases in cell volume was determined. Hypoxanthine and other salvagable purines restored the ability of dialyzed serum to support methotrexate-induced increases in cell volume, but the concentration of hypoxanthine required was somewhat greater than that available when undialyzed serum was used to supplement the basal growth medium. Although formation of giant cells required an exogenous purine, methotrexate-stimulated expression of heat-stable alkaline phosphatase or morphological differentiation to the STL phenotype was unaffected by hypoxanthine. Dipyridamole, an inhibitor of nucleobase and nucleoside transport (6-8), inhibited methotrexate-induced increases in cell volume and hypoxanthine transport indicating that uptake of exogenous hypoxanthine was required for giant cell formation. The results presented in this chapter indicate that the dialyzable factor required for giant cell formation was hypoxanthine.

B. Results

It is evident from data presented in Table 16 that increases in RNA and protein content per cell were less when BeWo cultures were exposed to 1 μM methotrexate in culture fluids containing dialyzed instead of untreated fetal bovine serum. In methotrexate-treated BeWo cultures maintained in medium supplemented with dialyzed serum, protein synthesis was inhibited less than RNA synthesis suggesting that decreased RNA synthesis blocked formation of giant cells. The increase in RNA content of BeWo cultures exposed to methotrexate for 72 hr in growth medium supplemented with dialyzed serum was only 13% of the increase observed in cultures maintained in medium supplemented with undialyzed serum. Rates of proliferation, RNA synthesis, or protein sythesis were not altered in drug-free cultures by dialysis of the serum supplement (Table 17). These results suggest that salvage-dependent RNA synthesis was required for methotrexate-induced increases in cell mass.

Table 16

Effects of Dialysis of Serum on RNA and Protein Content of 1 μM Methotrexate-treated BeWo cells

	RNA (μg/10^5 cells)		Protein (μg/10^5 cells)	
Time (hr)	Undialyzed FBS	Dialyzed FBS	Undialyzed FBS	Dialyzed FBS
0	21.2 ± 1.5		125 ± 12	
24	36.5 ± 2.7	29.1 ± 2.5[1]	224 ± 13	212 ± 12[2]
48	33.9 ± 1.6	29.0 ± 2.5[1]	249 ± 9	221 ± 11[1]
72	39.5 ± 0.7	23.5 ± 0.7[1]	299 ± 6	229 ± 13[1]

[1] *significantly different* ($p>0.05$) *from Undialyzed FBS.*

[2] *not significantly different* ($p>0.05$) *from Undialyzed FBS.*

BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and 24 and 72 hr later, culture fluids were replaced with growth medium containing 1 μM methotrexate supplemented with either dialyzed or undialyzed fetal bovine serum (FBS). At 24-hr intervals, cell number, RNA, and protein content per flask (mean±S.D., n=3 cultures from a representative experiment of a series of 3). were determined as described in Materials and Methods (section E).

Table 17

Effects of Dialysis of Serum on Proliferation Rate and RNA and Protein Content of Methotrexate-free BeWo Cells

	Serum Treatment	
	Undialyzed FBS	Dialyzed FBS
Mean Population Doubling Time (hr)	23.4 ± 7.3 (37)	24.3 ± 6.1[1] (37)
RNA (μg/10^5 cells)	29.7 ± 2.9 (9)	31.1 ± 3.2[1] (9)
Protein (μg/10^5 cells)	142 ± 28 (12)	141 ± 25[1] (12)

[1] *not significantly different* ($p > 0.05$)

BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with growth medium supplemented with either dialyzed or untreated fetal bovine serum, and cell numbers were determined electrically (Materials and Methods, section A). After 48-hr exposures, cell numbers and RNA and protein content were determined as described in Materials and Methods (sections A and E). Population doubling times were calculated from the number of cells at 24 and 72 hr.

Charcoal extraction removes purines by adsorption (9), and xanthine oxidase enzymatically converts hypoxanthine and xanthine to uric acid (10). It is evident from the data summarized in Table 18 that treating fetal bovine serum with charcoal or xanthine oxidase reduced methotrexate-induced increases in cell volume to levels observed when cells were exposed to drug in growth medium supplemented with dialyzed serum. These results suggest that hypoxanthine and xanthine were the exogenous factors required for formation of BeWo giant cells.

Table 18

Effects of Removing Purines from Serum on Methotrexate-induced Formation of Giant Cells

Serum Treatment	Modal Volume (pl/cell)
Untreated	6.22 ± 0.17
Dialyzed	3.58 ± 0.09
Charcoal-extracted	4.07 ± 0.02
Xanthine oxidase-treated	4.09 ± 0.06

Fetal bovine serum was treated as described in Materials and Methods (section A.1). BeWo cultures were established with 2 x 10^5 cells/T-25 flask in growth medium containing untreated serum, and after 24 hr, culture fluids were completely replaced with growth medium supplemented with variously treated sera, and cultures were incubated for an additional 24 hr. Culture fluids were then replaced again with growth medium supplemented with the appropriate serum containing 1 μM methotrexate. After 48-hr exposures, modal cell volume (average ± S.D., n = 3) was determined electrically as described in Materials and Methods (section B.1).

The purine content of fetal bovine serum was assessed by reversed-phase high performance liquid chromatography. Representative chromatography tracings for the separation of purines and nucleosides are shown in Figure 14. A chromatography system could not be developed that simultaneously separated purines and nucleosides. Hypoxanthine, xanthine, and uric acid were present in fetal bovine serum, and treatment of serum with xanthine oxidase converted all hypoxanthine and xanthine to uric acid (Figure 15). The purine content of several serum lots was determined, and those results are summarized in Table 19. The concentrations of uric acid, xanthine, and hypoxanthine present in different samples of fetal bovine serum (6 different lots from 4 companies) were relatively constant and were similar to concentrations for hypoxanthine, xanthine, and uric acid (50 and 75 μM, 92 μM, and 130 μM, respectively) previously reported (11, 12).

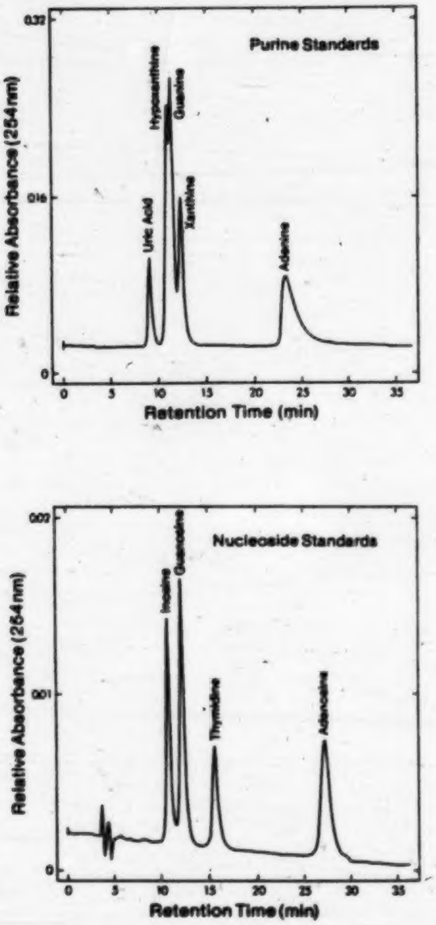

Figure 14. Separation of purines and nucleosides by reversed-phase high performance liquid chromatography. Purines were separated by isocratic elution of a Partisil PXS 5/25 ODS-3 column (Waters) with 50 mM phosphate (pH 4.6) at a flow rate of 1 ml/min. Nucleosides were isocratically eluted from the same column with 50 mM phosphate (pH 4.6) buffer:methanol (94:6) at a flow rate of 1 ml/min (Materials and Methods, section F.1).

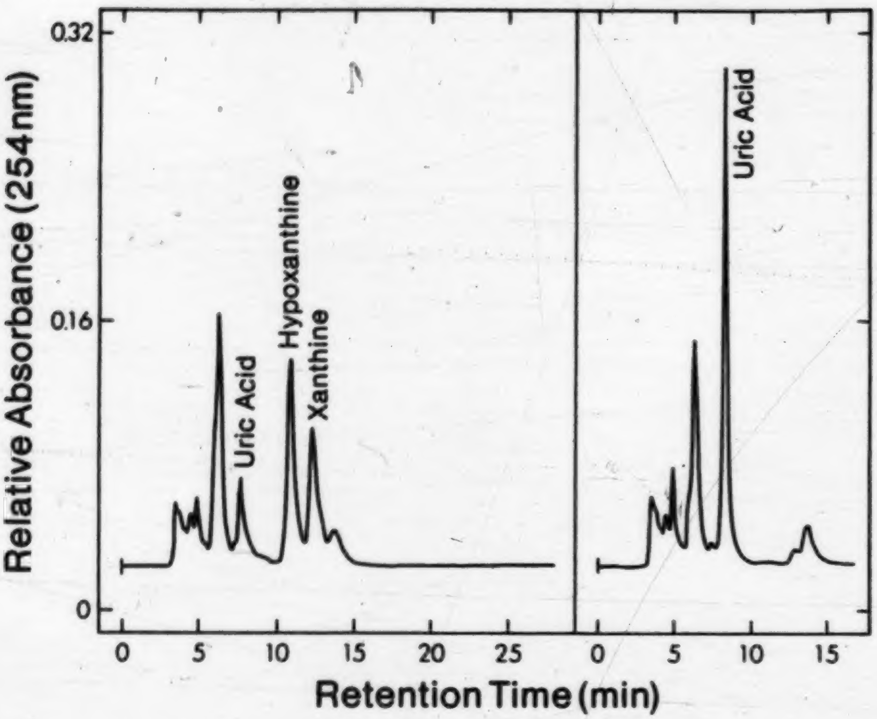

Figure 15. Effects of xanthine-oxidase treatment on purine content of fetal bovine serum. Purines in untreated (Panel A) and xanthine-oxidase treated (Panel B) fetal bovine serum were identified after serum samples were deproteinated by centrifugation through an Amicon (CF-25) membrane filter. The chromatography system was as described in Figure 14. Hypoxanthine, xanthine, and uric acid were identified by retention time, cochromatography with added standards, and hypoxanthine and xanthine were also identified by enzymatic shift to uric acid with xanthine oxidase as described in Materials and Methods (section F.1).

Table 19

Purine Content of Fetal Bovine Serum

Purine	Concentration (μM)
Hypoxanthine	53 ± 18 (6)[1]
Xanthine	86 ± 19 (6)
Uric Acid	139 ± 22 (6)
Guanine	2 ± 1 (6)
Adenine	< 2.5[2] (6)
Adenosine	< 0.5[2] (4)
Guanosine	< 0.2[2] (2)
Inosine	< 0.2[2] (2)

[1] *number of different serum lots*

[2] *limit of detection*

Purines and nucleosides present in fetal bovine serum were quantitated (mean ± S.D.) by reversed-phase high performance liquid chromatography as described in Figure 15.

The ability of purine nucleosides and bases to augment methotrexate-induced increases in cell volume was assessed to confirm that the dialyzable factor required for giant cell formation was a purine. It is evident from the concentration-effect relationship presented in Figure 16 that hypoxanthine restored the ability of dialyzed fetal bovine serum to support methotrexate-induced increases in cell volume. The concentration of exogenous hypoxanthine that yielded 50% of the maximal increase in cell volume was approximately 80 μM. Hypoxanthine did not alter the volume of drug-free cells. Furthermore, the presence of hypoxanthine (10^{-9} -10^{-3}) in the culture fluids of methotrexate-free cultures did not significantly alter proliferation rates (Table 20). These results indicate that the ability of hypoxanthine to augment methotrexate-induced increases in cell volume was not related to a nonspecific stimulation of cell growth.

The ability of individual purines and purine nucleosides to augment methotrexate-induced formation of giant cells was assessed by measuring changes in cell volume during exposure to drug in medium supplemented with dialyzed serum (Table 21). The relative ability of other purines to support giant cell formation was hypoxanthine > inosine > adenosine = guanosine > xanthine = uric acid = no purine supplement. With the exception of guanosine, the purines and purine nucleosides tested did not affect proliferation rates of methotrexate-free cultures. The number of doublings during the 48-hr exposure period (mean ± S.D., $n=3$ unless otherwise indicated) in the absence of additives was 2.29 ± 0.42 ($n=17$) and in the presence of additives were: guanosine (1.51 ± 0.02); hypoxanthine (2.14 ± 0.56, $n=9$); adenosine (2.00 ± 0.26); inosine (2.32 ± 0.17); xanthine (2.28 ± 0.19); and uric acid (2.20 ± 0.21).

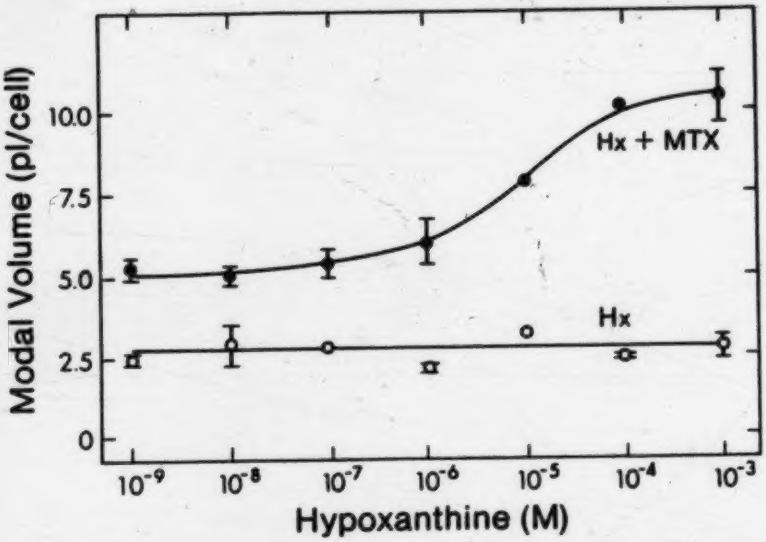

Figure 16. Effects of hypoxanthine on the modal volume of proliferating and methotrexate-treated BeWo cells cultured in growth medium supplemented with 10% dialyzed fetal bovine serum. BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with drug-free growth medium containing hypoxanthine (○) or growth medium containing 1 μM methotrexate and hypoxanthine (●) at the concentrations indicated. After 48-hr exposures, modal cell volumes (average±S.D., n=3) were determined as described in Materials as Methods (section B.1). The modal volumes (average±S.D.) of cells subjected to 48-hr exposures to 1 μM methotrexate in medium supplemented with undialyzed fetal bovine serum (10.3±1.3 pl/cell, n=9) or with dialyzed fetal bovine serum and excess hypoxanthine (>10^{-4} M, 10.1±0.6 pl/cell, n=11) were the same ($p>0.05$).

Table 20

Effects of Hypoxanthine on Proliferation Rates of BeWo Cells

Hypoxanthine (M)	Doubling Time (hr)
0	20.3 ± 3.2 (5)[1]
10^{-3}	19.7 ± 0.7[2] (4)
10^{-4}	19.4 ± 0.6[2] (4)
10^{-5}	20.5 ± 2.0[2] (4)
10^{-6}	20.6 ± 2.2[2] (5)
10^{-7}	21.3 ± 0.9[2] (3)
10^{-8}	22.5 ± 1.8[2] (3)
10^{-9}	23.1 ± 1.9[2] (3)

[1] *number of separate cultures*

[2] *not significantly different from population doubling time of hypoxanthine-free cultures* ($p > 0.05$)

BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with methotrexate-free growth medium containing hypoxanthine at the concentrations indicated. Cell numbers were determined 24 and 72 hr after cultures were initiated, and values are the population doubling times (±S.D.).

Table 21

Methotrexate-induced Formation of Giant Cells in Growth Medium Supplemented with Purines and Purine Nucleosides

	Modal Volume (pl/cell)	
Compound 200 μM	MTX-free	MTX-treated
None	3.66 ± 1.08 (33)[1]	5.58 ± 0.86 (12)
Adenosine[2]	4.20 ± 0.61 (3)	7.31 ± 0.95[4] (3)
Guanosine	4.96 ± 0.77 (3)	7.22 ± 0.95[4] (3)
Inosine	3.47 ± 0.23 (3)	8.72 ± 1.35[4] (3)
Hypoxanthine[3]	2.40 ± 0.10 (3)	10.95 ± 1.67[4] (9)
Xanthine	4.03 ± 0.25 (3)	5.10 ± 0.47[5] (3)
Uric Acid	4.30 ± 0.69 (3)	5.18 ± 0.22[5] (3)

[1] *number of separate determinations*

[2] *adenosine was tested in the presence of 1 μM 2'-deoxycoformycin*

[3] *hypoxanthine concentration was 100 μM*

[4] *significantly different from drug treated without purine supplement (*$p>0.05$*)*

[5] *not significantly different from drug treated without purine supplement (*$p>0.05$*)*

The basal growth medium was supplemented with 10% dialyzed fetal bovine serum. BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with methotrexate-free medium (MTX-free) or with medium containing 1 μM methotrexate; purine bases and nucleosides were present in culture fluids as indicated. After 48-hr exposures, modal cell volumes (average±S.D.) were determined electrically (Materials and Methods B.1).

The effects of hypoxanthine on methotrexate-induced expression of syncytiotrophoblastic markers by BeWo cells are summarized in Table 22. It is evident that increases in cell volume were dependent on a source of exogenous hypoxanthine, whereas differentiation to the STL phenotype or expression of heat-stable alkaline phosphatase were unaffected by hypoxanthine.

Table 22

Effects of Exogenous Hypoxanthine on Expression of Syncytiotrophoblastic Markers

	Dialyzed FBS		Dialyzed FBS + 100 μM Hypoxanthine	
Marker	Untreated	MTX-treated	Untreated	MTX-treated
Morphology	CTL	STL	CTL	STL
Cell volume	3.34 ± 0.72 (27)	6.49 ± 1.47 (13)	4.28 ± 1.45 (9)	10.96 ± 1.45 (9)
Alkaline phosphatase	12.9 ± 3.1 (9)	76.6 ± 14.1 (9)	19.7 ± 3.1 (9)	57.8 ± 18.9 (9)

BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with growth medium (dFBS) or medium (dFBS) containing 1 μM methotrexate with or without 100 μM hypoxanthine. After 48-hr exposures, expression of markers of the BeWo CTL-to-STL differentiative response were quantitated as described previously (Chapter V). The predominant morphology (> 90% of total cells) of cultures was determined with phase-contrast microscopy by scoring at least 100 cells/culture. Modal cell volume (pl/cell, average±S.D.) was determined electrically (Materials and Methods, section B.1), and the specific activity (nmol *p*-nitrophenol released/min/mg protein) of heat-stable alkaline phosphatase in crude extracts (mean±S.D., n=triplicate assays) was determined as described in Materials and Methods (section G).

To determine if uptake of hypoxanthine was required for formation of giant cells, the effects of dipyridamole, an inhibitor of nucleoside and nucleobase transport (6-8), on methotrexate-induced increases in BeWo cell volume and uptake of hypoxanthine were assessed. It is apparent from the results presented in Table 23 that dipyridamole or hypoxanthine, in combination or alone, did not effect the volume of proliferating BeWo cells, whereas dipyridamole inhibited hypoxanthine-dependent methotrexate-induced increases in BeWo cell volume. At the highest dipyridamole concentration tested (10 μM) increases in modal cell volume after 48-hr exposures to methotrexate were inhibited by 53% in comparison to increases in cell volume in the presence of methotrexate and hypoxanthine.

To determine if inhibition of methotrexate-induced increases in BeWo cell volume by dipyridamole was related to inhibition of nucleobase transport, the initial rate of hypoxanthine uptake by BeWo cells in the presence and absence of dipyridamole was measured using a centrifugal method (13). Uptake of nucleosides and bases by cultured cells is usually linear for 1 to 10 sec, and initial uptake velocities are assumed to reflect the rate of transport of the permeant into the cell (14).

It is evident from the data presented in Figure 17 that uptake of hypoxanthine by BeWo cells was less in the presence of dipyridamole. The rate of hypoxanthine uptake by dipyridamole-treated cells was inhibited approximately 82% in comparison to the rate of uptake by untreated cells. This observation indicates that mediated transport of hypoxanthine by BeWo cells was sensitive to inhibition by dipyridamole suggesting that the effects of dipyridamole on methotrexate-induced increases in cell volume were related to inhibition of hypoxanthine uptake.

Table 23

Effects of Dipyridamole and Hypoxanthine on BeWo Cell Volume

Additives	MTX-free	MTX-treated
none	4.91 ± 0.57	5.96 ± 0.31
Hx + 10 μM dipyridamole	4.57 ± 0.59	7.87 ± 0.64
Hx + 5 μM dipyridamole	4.57 ± 0.62	9.56 ± 1.08
Hx + 1 μM dipyridamole	4.61 ± 0.37	9.66 ± 0.94
Hx	5.21 ± 0.45	10.02 ± 0.65

BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with methotrexate-free medium (dFBS) or medium (dFBS) containing 1 μM methotrexate; 100 μM hypoxanthine and dipyridamole were present in culture fluids as indicated. After 48-hr exposures, modal cell volume (average ± S.D., n=6) was determined electrically as described in Materials and Methods (section B.1). The population doubling times (mean ± S.D., n=3) of methotrexate-free BeWo cultures in the presence of 100 μM hypoxanthine (19.3 ± 1.5 hr) or 100 μM hypoxanthine and 10 μM dipyridamole (19.5 ± 0.9 hr) were the same ($p > 0.05$) as that of cultures maintained in the absence of these additives (19.8 ± 2.3 hr).

Although the concentrations of dipyridamole used for this study were nontoxic to proliferating BeWo cells (Table 23), dipyridamole alters cellular processes of cultured cells that are unrelated to nucleobase and nucleoside transport (15). Therefore, these experiments do not eliminate the possibility that dipyridamole had effects on BeWo cells other than inhibition of hypoxanthine transport that resulted in inhibition of methotrexate-induced increases in cell volume.

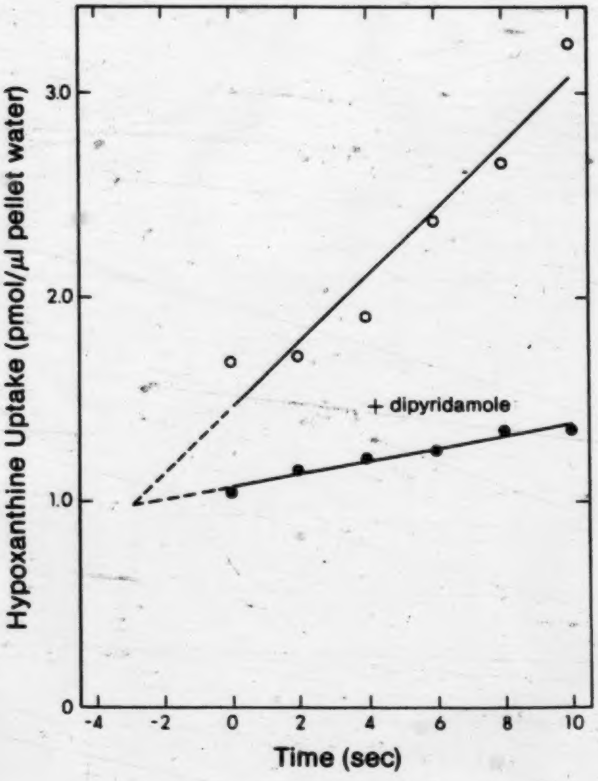

Figure 17. Effects of dipyridamole on uptake of hypoxanthine by BeWo cells. Uptake of hypoxanthine (10.7 μM) by BeWo cells was measured as described in Materials and Methods (section D) in the presence (●) and absence (○) of 10 μM dipyridamole. Total pellet and extracellular volumes were determined in parallel experiments by equilibration of BeWo cells with [^{3}H]water and [^{14}C]sucrose. The extracellular volume (0.36±0.04 μl, n=4) was 12% of the total volume of the cell pellet (water volume was 2.96±0.21 μl/cell pellet, n=4), and the extracellular hypoxanthine volume (0.54 μl) determined by extrapolation of the rate of hypoxanthine uptake in the presence and absence of dipyridamole to time zero was 18% of the total water volume of the cell pellet.

C. Summary

Although RNA and protein contents of BeWo cells exposed to 1 μM methotrexate in a hypoxanthine-depleted growth medium increased modestly, maximal increases in cell mass required dialyzable factor(s) present in fetal bovine serum. The dialyzable factor was hypoxanthine since: (1) specific methods for removing purines (charcoal extraction and xanthine-oxidase treatment) decreased the ability of serum to support methotrexate-induced increases in cell size; (2) exogenous hypoxanthine restored the ability of dialyzed fetal bovine serum to support maximal increases in cell volume; and (3) hypoxanthine as well as xanthine were present in untreated fetal bovine serum. Although hypoxanthine was the most efficient source of the purine ring, inosine, adenosine, and guanosine also augmented methotrexate-induced increases in cell volume in growth medium supplemented with dialyzed serum. The concentrations of hypoxanthine and xanthine present in untreated fetal bovine serum were less than that required to restore capacity of dialyzed serum to support maximal increases in cell size, but other unidentified dialyzable factors (reduced folates, small amounts of other purines) or protein bound purines that were slowly exchangable could explain this difference.

Mediated transport of hypoxanthine was apparently required for formation of giant cells as dipyridamole, an inhibitor of nucleoside and base transport, inhibited increases in cell volume and was shown to inhibit uptake of hypoxanthine from culture fluids. Uptake of hypoxanthine was not completely inhibited by dipyridamole at the concentrations tested in this work; thus, it was likely that less than complete inhibition of hypoxanthine-dependent increases in cell volume were related to residual influx of hypoxanthine in the presence of dipyridamole.

During the first 48 hr of continous exposure to methotrexate in growth medium supplemented with untreated serum, increases in RNA content required 26.2 ± 3.4 nmol of purine (assuming a purine:pyrimidine ratio of 1:1 and an average molecular weight of 300 for a RNA-incorporated purine nucleotide). The amount of hypoxanthine available from the growth medium (26.5 ± 9.0 nmol) was comparable, suggesting that salvage of hypoxanthine was rate limiting for RNA synthesis during the CTL-to-STL differentiative response.

A requirement for serum factors to express differentiated function was previously shown with mouse 3T3 cells. Adipocyte conversion of 3T3 cells was interrupted by biotin deficiency. When biotin, a cofactor required for carboxylation during fatty acid synthesis (16), was removed from the growth medium, 3T3 cells did not accumulate triglyceride (17,18). Biotin deficiency inhibited 3T3 cell *de novo* synthesis of long-chain fatty acids required for triglycerides (18) Other markers of mature adipocyte function were expressed under conditions of biotin deficiency; therefore, biotin was not a regulator of the cytodifferentiative response, but was simply an extrinsic cofactor required for biosynthetic pathways activitated during the response (18).

In conclusion, results presented in this Chapter indicate that hypoxanthine was an exogenous factor required for methotrexate-induced formation of giant cells. However, morphological differentiation to the STL phenotype or expression of heat-stable alkaline phosphatase were not dependent on an exogenous source of hypoxanthine. Dialysis of serum supplements primarily inhibited increases in RNA content of BeWo cells during exposure to methotrexate suggesting that hypoxanthine was required for continued RNA synthesis in the presence of methotrexate. Thus, hypoxanthine was apparently a factor required for continued growth of STL cells and was not a regulatory factor involved in controlling the CTL-to-STL differentiative response.

D. References

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VII. Mechanism of Action of Methotrexate During Induction of the CTL-to-STL Cytodifferentiative Response

A. Introduction

Although the BeWo cell line was derived from a choriocarcinoma obtained from a patient who had developed resistance to methotrexate chemotherapy, cultured BeWo cells were sensitive to the antiproliferative effects of methotrexate as evidenced by an IC_{50} value for inhibition of proliferation of 50 nM (see Chapter V, section B.1). The antiproliferative and cytotoxic effects of methotrexate have been attributed to disruption of one-carbon metabolism that is dependent on tetrahydrofolate cofactors (1-5). ^{5}N,^{10}N-Methylenetetrahydrofolate, ^{10}N-formyltetrahydrofolate, and ^{5}N-methyltetrahydrofolate contribute one-carbon units required for the methyl group of thymidylate (6), C-2 and C-8 of the purine ring (7), the methyl group of methionine (8), the α-carbon of glycine (9), and the β-carbon of serine (10) (see Figure 18). Methotrexate disrupts folate-dependent biosynthetic pathways by blocking formation of tetrahydrofolate (11). During the synthesis of thymidylate, ^{5}N,^{10}N-methylenetetrahydrofolate serves both as a one-carbon and as an electron donor in the transfer of a methyl group to deoxyuridylate. The loss of a hydride ion from the pteridine ring results in conversion of tetrahydrofolate to dihydrofolate (12). Since dihydrofolate is not an active coenzyme and tetrahydrofolate cofactors are present in cells at catalytic concentrations, continued thymidylate synthesis in the presence of methotrexate leads to depletion of folate cofactors (see Chapter II). Reactions of purine, pyrimidine, and amino acid synthesis utilize tetrahydrofolate cofactors, and exposure to methotrexate can result in inhibition of DNA, RNA, and protein synthesis. Biochemical mechanisms of resistance to methotrexate include elevated levels of dihydrofolate reductase, decreased methotrexate uptake, and decreased binding of methotrexate to dihydrofolate reductase (14-16).

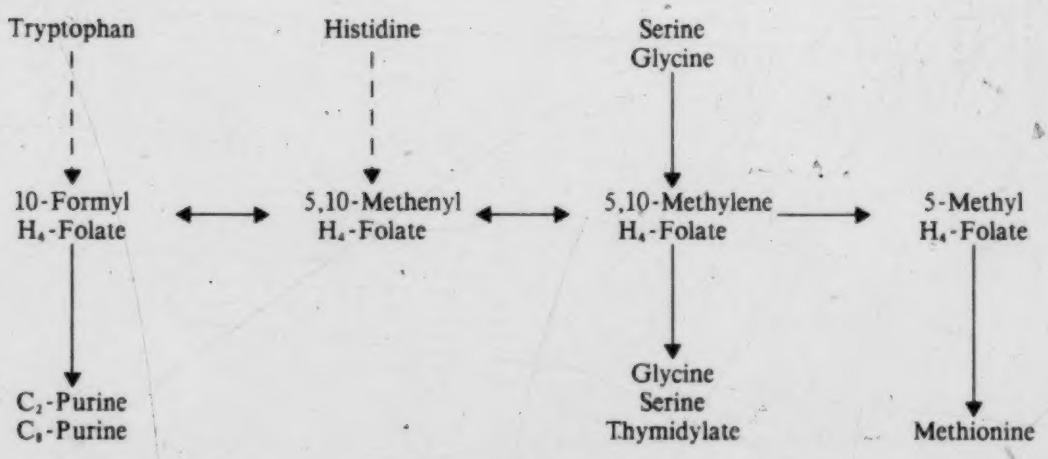
Tryptophan
Histidine
Serine
Glycine
10-Formyl
H_4-Folate
5,10-Methenyl
H_4-Folate
5,10-Methylene
H_4-Folate
5-Methyl
H_4-Folate
C_2-Purine
C_8-Purine
Glycine
Serine
Thymidylate
Methionine

Figure 18. The source and metabolic fate of one-carbon units, and interconversions of tetrahydrofolate (H_4-Folate) cofactors. Leucovorin (^{5}N-formyltetrahydrofolate, not shown) can be converted to either ^{10}N-formyltetrahydrofolate or ^{5}N,^{10}N-methenyltetrahydrofolate. Adapted from reference 13.

There is little doubt that thymidine and purine starvation are the primary cytotoxic effects of methotrexate, but methotrexate also disrupts cellular processes not directly related to inhibition of dihydrofolate reductase (see Chapter 2). In isolated enzyme systems, thymidylate synthetase and other folate-dependent enzymes are inhibited by methotrexate, and polyglutamate forms of methotrexate are more potent inhibitors than the parent drug. In studies with cultured cells, methotrexate has also been demonstrated to disrupt several metabolic processes secondary to inhibition of dihydrofolate reductase. The importance of these effects to methotrexate cytotoxicity is not known. The complex pharmacology and the biological response of BeWo cells to methotrexate has lead to the suggestion that methotrexate may induce the CTL-to-STL differentiative response through a novel mechanism of action involving sensitivity of a number of different target sites or differential depletion of tetrahydrofolate cofactor pools (17).

Experiments were undertaken to assess the sensitivity of BeWo cells to the primary biochemical effects of methotrexate and to determine if these effects were responsible for stimulating expression of syncytiotrophoblastic markers. Although the activity of dihydrofolate reductase and its inhibition by methotrexate can be assessed directly by measuring reduction of [^{3}H]dihydrofolate, inhibition of biochemical processes dependent on tetrahydrofolate cofactors are more commonly determined in studies with intact cells. The effects of methotrexate on the apparent intracellular activity of thymidylate synthetase and incorporation of formate into DNA, RNA, and protein were assessed to determine if folate-requiring biosynthetic pathways were inhibited. In addition, the DNA content of BeWo cells was measured by flow cytometry to determine if exposure to methotrexate resulted in an S-phase block in progression through the cell cycle. When BeWo cells were exposed to methotrexate at concentrations supramaximal for inhibition of proliferation, the apparent rate of thymidylate synthesis was decreased within minutes and the rates of [^{14}C]formate incorporation into DNA, RNA, and protein were inhibited approximately 95%. Cells accumulated in G_1 and early S phases of the cell cycle, indicating that continous exposure to methotrexate inhibited DNA synthesis.

To determine if inhibition of dihydrofolate reductase was the basis for induction of the BeWo CTL-to-STL differentiative response by methotrexate, the capacity of thymidine, hypoxanthine, and leucovorin to block the drug's effects were assessed. If methotrexate was acting by a mechanism unrelated to depletion of tetrahydrofolate cofactors, then coadministration of a purine and thymidine or a source of tetrahydrofolate should not alter methotrexate-induced cytodifferentiation. Coadministration of hypoxanthine and thymidine or leucovorin abolished both the cytodifferentiative and the antiproliferative effects of methotrexate. Differential effects of hypoxanthine and thymidine alone during exposure to methotrexate indicated that either purine or thymidylate starvation inhibited BeWo cell proliferation and suggested that induction of the cytodifferentiative response was a result of thymidylate starvation. To test this hypothesis, the effects of a specific inhibitor of thymidylate synthetase on expression of syncytiotrophoblastic markers by BeWo cells was investigated. Fluorodeoxyuridine, which has previously been shown to induce morphological differentiation of BeWo cells,[5] inhibited proliferation, stimulated increases in cell volume and expression of heat-stable alkaline phosphatase, and induced differentiation to the STL phenotype. These effects were blocked by coadministration of thymidine.

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[5] Fluorodeoxyuridine (4 μM) induces BeWo cells to differentiate to the STL phenotype (99% of total cells are giant cells, 8% of which are multinucleated), personal communication, Dr. S. J. Friedman.

B. Results

1. Incorporation of deoxyuridine and thymidine

The effect of methotrexate on thymidylate synthesis was assessed by comparing incorporation of [6-^{3}H]deoxyuridine and [*methyl*-^{3}H]thymidine into acid-precipitable material of BeWo cultures at various intervals after initiating drug exposures (Figure 19). The rates of incorporation of thymidine and deoxyuridine by drug-free cultures were not significantly different ($p>0.05$) over the 2-hr exposures used in the experiment of Figure 19 (data not shown). Utilization of exogenous thymidine by methotrexate-treated BeWo cultures increased relative to that of drug-free cultures, reaching a peak 45 min after exposure to methotrexate began. Conversely, utilization of deoxyuridine by methotrexate-treated cultures decreased relative to that of drug-free cultures, suggesting that tetrahydrofolate had become rate-limiting for thymidylate synthesis.[6] The decline in incorporation of thymidine seen after 45 min suggests that purines may have become limiting for continued synthesis of nucleic acids. Inhibition of thymidylate synthesis following exposure to methotrexate is consistent with substrate limitation of the tetrahydrofolate cofactors that are required for continued activity of thymidylate synthetase. These results are in agreement with those of an earlier study, which demonstrated increased incorporation of exogenous thymidine, and decreased incorporation of deoxyuridine, in BeWo cells subjected to prolonged exposures (up to 100 hr) to methotrexate (18).

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[6] Assuming that the activity of thymidine kinase remained constant during exposures to methotrexate and that the extracellular concentrations of precursors used in the experiment of Figure 19 did not perturb intracellular deoxynucleotide pools.

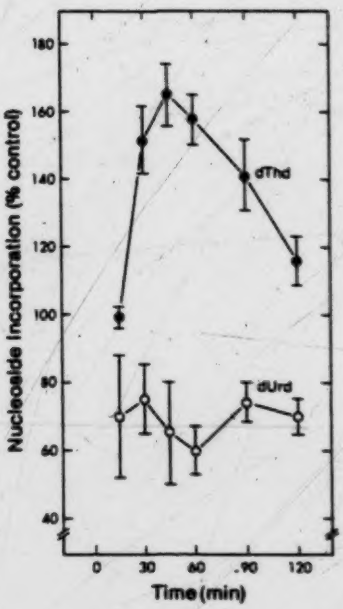
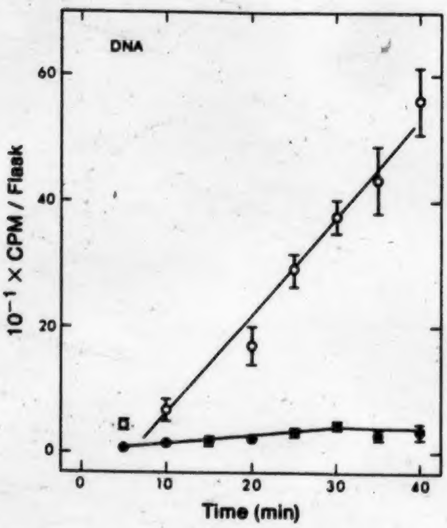


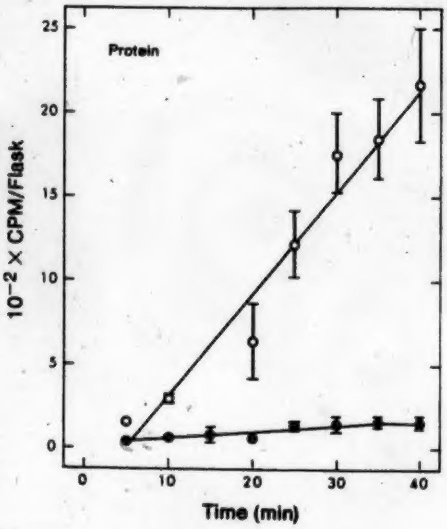
Figure 19. Effects of methotrexate on incorporation of deoxyuridine (dUrd) and thymidine (dThd) into acid-precipitable material of BeWo cells. BeWo cultures were established with 4 x 10^4 cells/glass scintillation vial, and after 24 hr, culture fluids were replaced with drug-free growth medium (dFBS) or medium (dFBS) containing 1 μM methotrexate. Thereafter, at the time intervals indicated, 1 ml of medium (dFBS) with or without 1 μM methotrexate and containing [6-^{3}H]deoxyuridine (○, 1 μCi/culture, final concentration 22 nM) or [*methyl*-^{3}H]thymidine (●, 1 μCi/culture, 6.7 nM) was added to each culture. Intervals of incorporation were ended after 10 min by aspiration of the culture fluids and acid precipitation with 0.2 N perchloric acid as described in Materials and Methods (section C.1). Each symbol represents the mean (±S.D., n=3) of cpm incorporated into methotrexate-treated cultures as a percentage of that incorporated into drug-free cultures (dThd, 5600 cpm; dUrd, 4980 cpm).

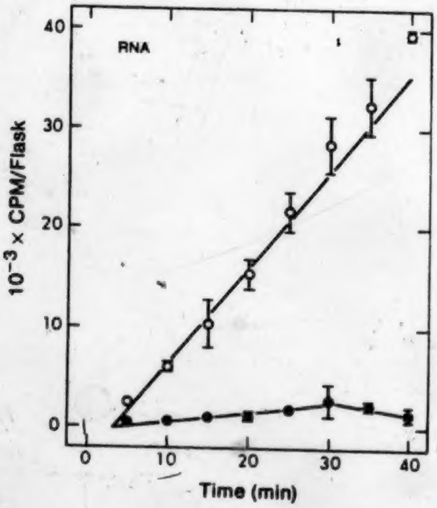
2. Formate incorporation

Formate is utilized as a one-carbon source in folate-requiring biosynthetic pathways for formation of C-2 and C-8 of the purine ring, the methyl group of thymidylate, and the folate-requiring pathways of amino acid synthesis. In the experiments of Figure 20, utilization of formate was assessed in BeWo cultures subjected to 1-hr exposures to 1 μM methotrexate. The relative amounts (percent of total counts) of formate incorporated into RNA, DNA, and protein, respectively, by untreated BeWo cells were 93.1%, 5.5%, and 1.4%. In drug-treated cultures, the rates of formate incorporation into DNA, RNA, and protein were inhibited approximately 95%, suggesting that tetrahydrofolate had become limiting for formation of folate cofactors required for thymidylate, purine, and folate-requiring amino acid synthesis.

Figure 20. Effects of methotrexate on incorporation of [^{14}C]formate into DNA, RNA, and protein of BeWo cells. BeWo cultures were established with 2 x 10^5 cells/60 mm tissue culture dish, and after 24 hr, culture fluids were replaced with drug-free growth medium (dFBS) or medium (dFBS) containing 1 μM methotrexate. After 1 hr, intervals of incorporation were initiated by adding 250 μl of medium (dFBS) containing [^{14}C]formate (41.7 μCi/culture, final concentration 0.17 mM) to each culture. Intervals of incorporation were ended at the times indicated by aspirating the culture fluids, washing with 0.15 M NaCl (5 ml), and extracting for 20 min with 0.2 M perchlorate (5 ml) at 4°. RNA, DNA, and protein were separated by the hydrolysis procedures described in Materials and Methods (section C.2). Each symbol represents the mean (±S.D., n=3) of radioactivity incorporated into DNA, RNA, and protein of drug-free (○) or methotrexate-treated (●) cultures.


DNA
10^{-1} × CPM / Flask
60
40
20
0
0
10
20
30
40
Time (min)


Protein
10^{-2} × CPM/Flask
25
20
15
10
5
0
0
10
20
30
40
Time (min)


RNA
10^{-3} × CPM/Flask
40
30
20
10
0
0
10
20
30
40
Time (min)

3. Cellular DNA content

The effects of methotrexate on incorporation of nucleic acid precursors demonstrated that *de novo* purine and thymidylate synthesis were inhibited in methotrexate-treated BeWo cells. Although a result of inhibition of these biosynthetic pathways should be inhibition of DNA synthesis, the CTL-to-STL cytodifferentiative response is accompanied by the appearance of multinucleated cells suggesting increases in DNA content per cell. To determine if exposure to methotrexate resulted in an S-phase block in progression through the cell cycle, the DNA content of ethanol-fixed BeWo cells was measured by flow cytometric analysis of mithramycin fluoresence.

In theory, the intensity of emitted flourescence can be measured on an absolute scale; in practice, relative flouresence is measured to obtain DNA distributions (20). Because relative fluorescence varies with staining conditions, and may be altered by drug-treatment, DNA distributions of different cell suspensions must be normalized to allow quantitative comparisons. The most reliable procedure consists of addition of a biological standard of constant DNA content, usually chick erythrocytes, to cell suspensions before staining (20). An alternate procedure was required in this study because the DNA content of G_1 BeWo cells was so much greater that that of chick erythrocytes (ca. 3.5-fold) that small variations in peak location, probably caused by sampling error, lead to considerable variations in the G_1 to chick cell peak ratio (data not shown). Therefore, cells from drug-free and methotrexate-treated cultures were mixed before fixing and staining to determine if exposure to methotrexate altered the relative fluorescence intensity of mithramycin-stained cells (Table 24). Because G_1 distributions appeared identical for drug-free and methotrexate-treated BeWo cells, in subsequent experiments DNA distributions were translocated so that the G_1 peak was in channel number 60, and this relative DNA content was defined as the 2N DNA content of BeWo cells.

The relative DNA contents of drug-free and methotrexate-treated BeWo cells are illustrated in Figure 21. Continous exposure to 1 μM methotrexate resulted in a gradual decrease

Table 24

Effects of Methotrexate on the Relative Fluoresence Intensity of G_1 BeWo Cells

	G_1 Distribution	
Untreated:MTX-treated	Peak Position	Half-height Width
1:0	75 ± 1	9 ± 1
3:1	74 ± 2	11 ± 1
2:1	73 ± 4	10 ± 1
1:1	76 ± 2	12 ± 1
0:1	76 ± 3	11 ± 2

BeWo cultures were established with 1 x 10^6 cells/T-75 flask, and after 72 hr, culture fluids were replaced with drug-free growth medium (dFBS) or medium (dFBS) containing 1 μM methotrexate. After 48-hr exposures, proliferating (Untreated) and methotrexate-treated (MTX) cultures were disaggregated by trypsinization, and cell suspensions were mixed in the numerical ratios indicated, fixed with ethanol, and stained with mithramycin for analysis with a Coulter EPICS V flow cytometer as described in Materials and Methods (section E.1). Relative fluorescence intensities (mean±S.D., n=3) are presented for the location of the modal channel of the G_1 peak and the width of the G_1 peak at half its height.

in the relative number of cells with a 4N DNA content and a concommitant increase in the number of cells in early S phase. Thus, although division of cells possessing a 4N DNA content continued, progression of cells through S phase of the cell cycle was inhibited during exposure to methotrexate, indicating that DNA synthesis was inhibited in methotrexate-treated cells. The effect of methotrexate on progression of BeWo cells through the cell cycle was quantitated by compartmentalizing DNA distributions into G_1, S, and G_2-M phases of the cell cycle (22). The model for parametric analysis (PARA2) assumed that G_1 cells formed a Gaussian distribution centered around a DNA content of 2N and that G_2-M cells formed a second Gaussian distribution centered around a DNA content 1.94-fold that of G_1 cells.[7] The standard deviations of the Gaussian distributions were determined from the steepest aspect of the curves.[8] S phase was modeled as a continous distribution between the G_1 and G_2-M peaks, and was subdivided into 4 equal compartments (S_1, S_2, S_3, S_4). The interfaces between S phase and G_1 or G_2 were determined by assuming that cells with 2N and 4N DNA contents were symmetrically distributed around their respective modes with a standard deviation determined from the steepest aspect of the curve.

Analyses of the DNA distributions of Figure 21 is presented in Figure 22. In the absence of methotrexate, the relative number of cells in each compartment of the cell cycle remained relatively constant over time with about 45% of cells in S phase. After initiation of methotrexate exposure, there was a transient increase, peaking at 6-7 hr, in the relative number of cells in G_1 of the cell cycle. With continued exposure to methotrexate (> 8 hr) the G_1 cells subsequently entered S phase, but apparently did not proceed through the cell cycle since there was accumulation of cells in early S phase (S_1 and S_2) as G_1 cells disappeared. The decline in

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[7] It would be expected that the G_2-M peak is centered at a channel number exactly twice that of the G_1 cells, but this is not experimentally observed (23).

[8] For DNA distributions (as presented in Figure 19), the "left" half of the G_1 peak and the "right" half of the G_2-M peak are the steepest aspects of the curves. The other halves of the G_1 and G_2-M peaks overlap with early and late S-phase, respectively; thus, the standard deviation of the "inner" half of each of these peaks is greater than that of the "outer" half.

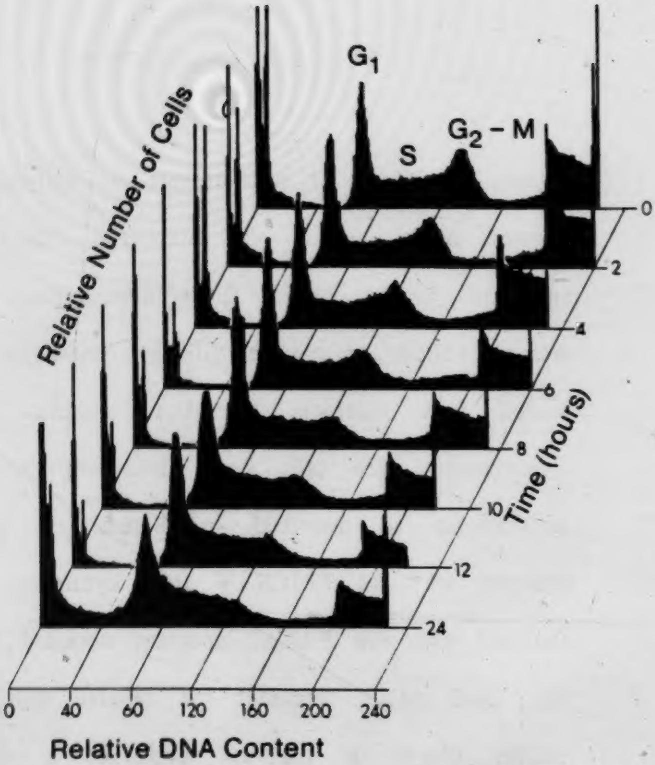
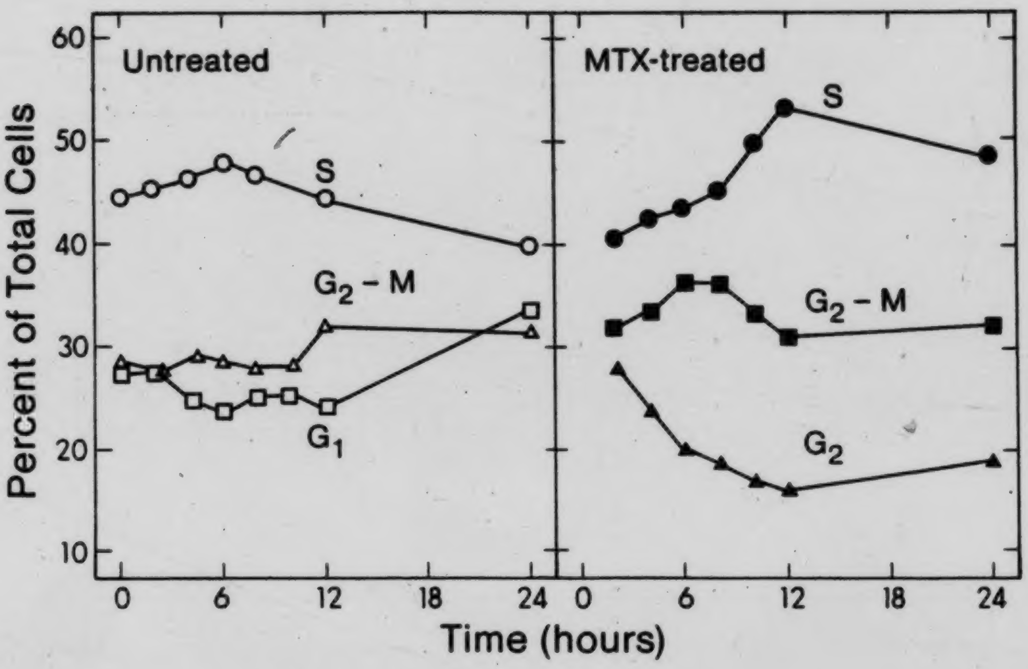
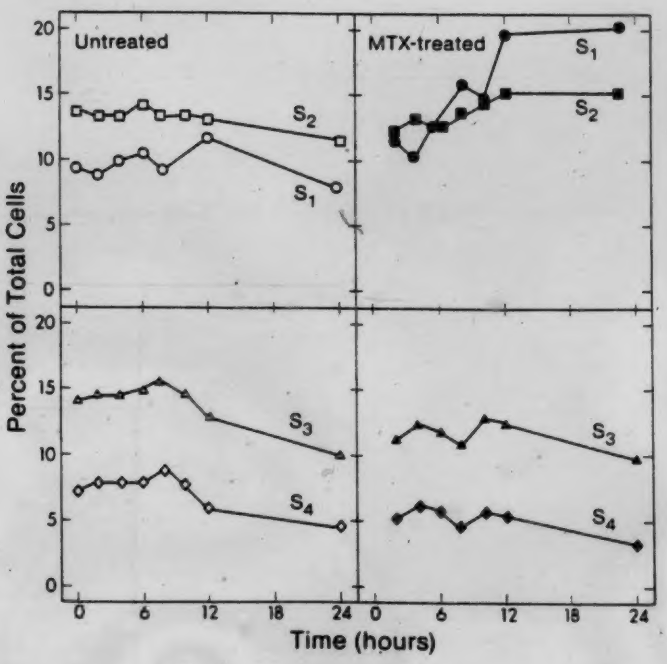


Figure 21. Effects of methotrexate on DNA content of BeWo cells. BeWo cultures were established with 1 x 10^6 cells per T-75 flask, and after 72 hr, culture fluids were replaced with growth medium (dFBS) containing 1 μM methotrexate. At graded time intervals thereafter, cultures were disaggregated by trypsinization, and cells were pelleted, suspended in 0.15 M NaCl, fixed with ethanol, and stained with mithramycin as described in Materials and Methods (section E.1). Flourescence distributions representing relative DNA content were obtained with an EPICS V flow cytometer and were smoothed with the least squares spline algorithim of the PARA1 program; G_1 peaks were translocated to channel 60, and the areas of the 2N to 4N region (channels 40 to 160) were normalized to a constant value. The 2N to 4N distribution represents the fluorescent intensities of 50,000 cells. Light scattering approaches (21) to characterize the particles with 4N and greater DNA content are presented in Appendix 2.

Figure 22. Effects of methotrexate on cell-cycle progression of BeWo cultures. BeWo cultures were established with 1 x 10^6 cells/T-75 flask, and after 72 hr, culture fluids were replaced with drug-free growth medium (dFBS) or medium (dFBS) containing 1 μM methotrexate. At graded time intervals thereafter, cultures were disaggregated by trypsinization, and cells were fixed with ethanol and stained with mithramycin as described in Figure 19. DNA distributions were obtained with an EPICS V flow cytometer, and distributions were analyzed with the PARA2 program obtained from Coulter Electronics Inc. Data points represent the relative number of cells present in various phases of the cell cycle of a single methotrexate-treated (closed symbols) or proliferating (open symbols) culture. The population doubling time of drug-free cultures in this experiment was 26.3 hr, and there was no proliferation in methotrexate-treated cultures. Upper panel: G_1 (□), S (○), or G_2-M (△). Lower panel: S_1 (○), S_2 (□), S_3 (△), S_4 (◊).


Untreated
MTX-treated
Percent of Total Cells
Time (hours)
S
G2 - M
G1
S
G2 - M
G2
60
50
40
30
20
10
0
6
12
18
24
0
6
12
18
24


Untreated
MTX-treated
Percent of Total Cells
Time (hours)
S2
S1
S1
S2
S3
S4
S3
S4
20
15
10
5
0
0
6
12
18
24

the number of cells in G_1 suggested that, although cells were initially blocked at the G_1-S phase transition by methotrexate treatment, there was, after 6-8 hr exposures, limited recovery of DNA synthetic capacity. The relative numbers of cells in the 6 compartments of the cell cycle after 48-hr exposures to methotrexate were not significantly different from those after 24-hr exposures (data not shown).

The validity of the mathematical model utilized for quantitative cell cycle analysis was graphically tested by comparing "raw" data with distributions generated from the "best-fit" parameters determined for G_1, the four compartments of S, and G_2-M phase (Figure 23). The model-generated DNA distributions were relatively similar in shape to the experimental DNA distributions, indicating that the model yielded parameters for DNA distributions that closely approximated the number of cells actually present in each compartment of the cell cycle. The only poor fit between the model and the data occurred with DNA distributions obtained from cells that had been exposed to methotrexate for 24 hr or longer, when the relative number of cells in the G_2-M peak was diminished and the G_2-M peak was poorly approximated by a symmetrical Gaussian distribution.

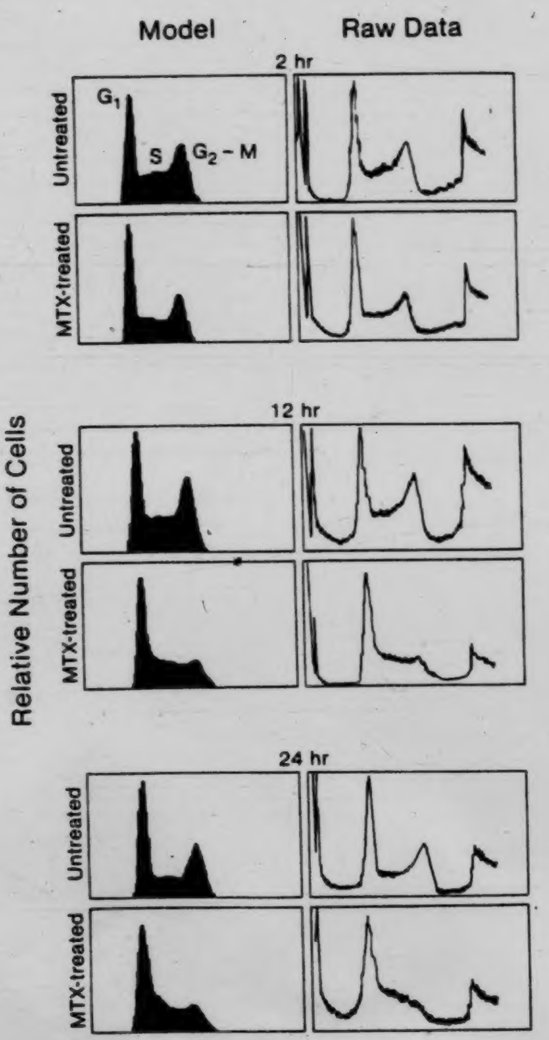


Figure 23. Graphical test of the validity of the PARA2 analysis program. Hypothetical DNA distributions were generated with the PARA2 program using the assumptions of the model and the best-fit parameters for the 6 compartments of the cell cycle determined for the analysis of Figure 22. For comparison, the raw data was plotted on the same scale.

4. Effects of protective agents

Previous experiments (sections B.1-B.3) indicated that exposure to methotrexate disrupted BeWo cell folate-dependent one-carbon metabolism. To determine if inhibition of dihydrofolate reductase and concommitant depletion of cellular thymidylate and purines was the effect of methotrexate responsible for inducing the CTL-to-STL differentiative response, protection experiments were undertaken. The toxic effects of methotrexate in mammalian cell cultures can be reduced or prevented by the addition of thymidine, a purine, and, in some cases, glycine (1, 24-27) or reduced folates (25-27). Leucovorin (^{5}N-formyltetrahydrofolate) can be converted to ^{5}N,^{10}N-methenyltetrahydrofolate or ^{10}N-formyltetrahydrofolate and apparently replenishes pools of reduced folate cofactors. (28). Hypoxanthine and thymidine are salvagable end-products of folate requiring biosynthetic pathways (6, 7). For all protection experiments presented in this Chapter, BeWo cells were exposed to methotrexate in growth medium supplemented with dialyzed fetal bovine serum to avoid interference from exogenous metabolites[9]

The thymidine and hypoxanthine requirements for BeWo cell proliferation in the presence of methotrexate were assessed to determine if depletion of these metabolites resulted in inhibition of proliferation. Neither hypoxanthine nor thymidine alone protected cells against the antiproliferative effects of methotrexate. However, coadministration of hypoxanthine and thymidine blocked the antiproliferative effects of 1 μM methotrexate. The concentration-effect relationships for protection by one precursor in the presence of an excess of the other differed by an order of magnitude. The PC_{50}[10] of hypoxanthine in the presence of excess thymidine (50 μM, Figure 24A) was ten-fold the PC_{50} of thymidine in the presence of excess hypoxanthine (5 μM, Figure 24B). These results indicate that methotrexate inhibited both *de novo* purine and

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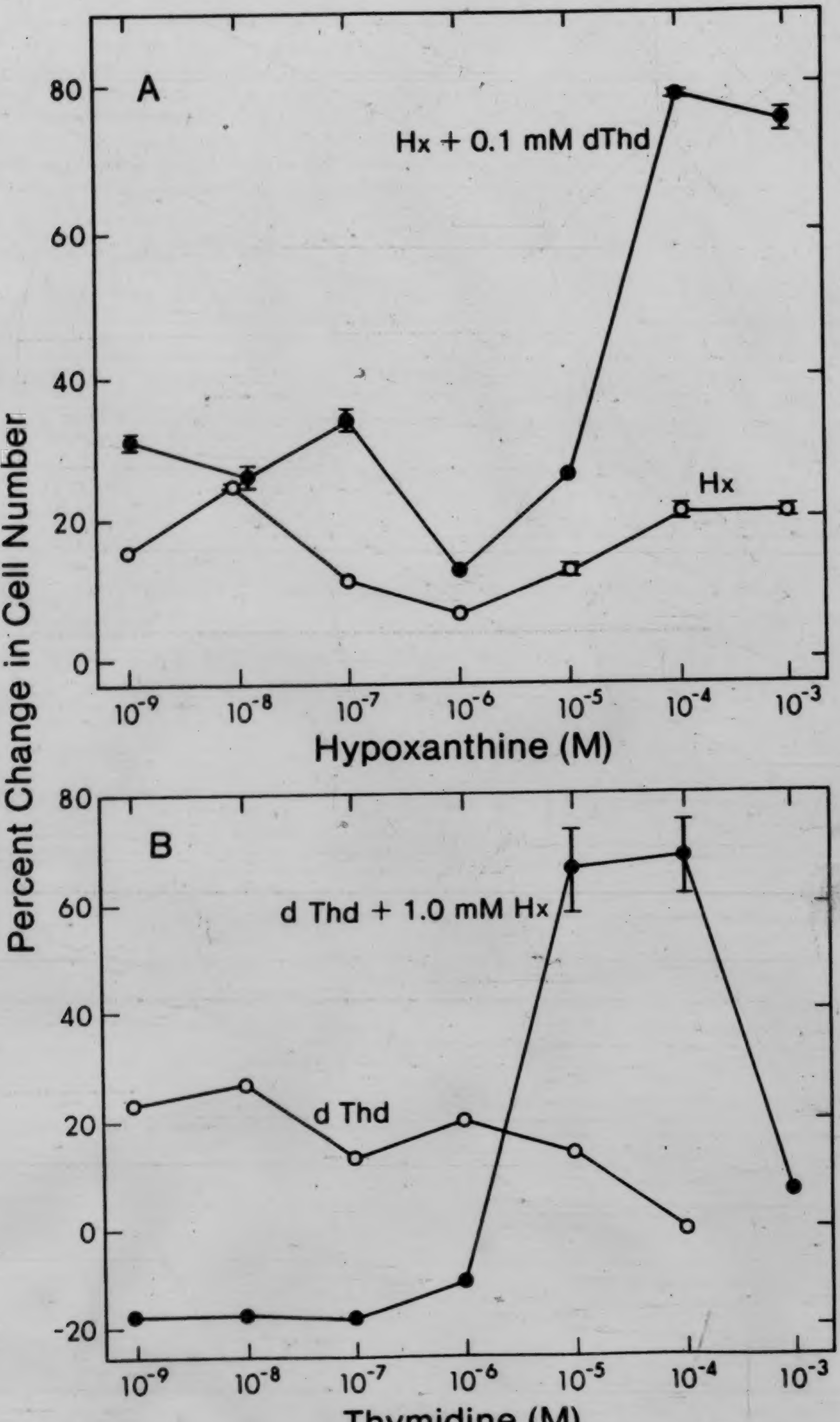
[9] The basal growth medium (RPMI medium 1640) contains 0.1 mM methionine, 0.13 mM glycine, 0.29 mM serine, and 2.3 μM folic acid, but does not contain reduced folates, nucleobases, or nucleosides.

[10] The PC_{50} is the protective concentration of one metabolite required for half-maximal increases in cell number in the presence of methotrexate and excess concentrations of the second metabolite.

Figure 24. Effects of thymidine and hypoxanthine on proliferation of BeWo cells exposed to 1 μM methotrexate. BeWo cultures were established with 2×10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with additive-free growth medium (dFBS) or medium (dFBS) containing 1 μM methotrexate plus thymidine and hypoxanthine at the concentrations indicated. After 48-hr exposures, cell numbers (mean ± S.D.) were determined with an electrical particle counter as described in Materials and Methods (section B.1). **A:** BeWo cells were exposed to 1 μM methotrexate and graded concentrations of hypoxanthine in the presence (●) and absence (○) of 0.1 mM thymidine. **B:** BeWo cells were exposed to 1 μM methotrexate and graded concentrations of thymidine in the presence (●) and absence (○) of 1.0 mM hypoxanthine. Data points represent the percent change in cell number (mean ± S.D., n=3), except that the open symbols in panel B are values from a single determination. The percent change in cell number was defined as: 100 x

$$\frac{\text{(treated cells/flask at 72 hr)} - \text{(control cells/flask at 24 hr)}}{\text{(control cells/flask at 72 hr)} - \text{(control cells/flask at 24 hr)}}$$

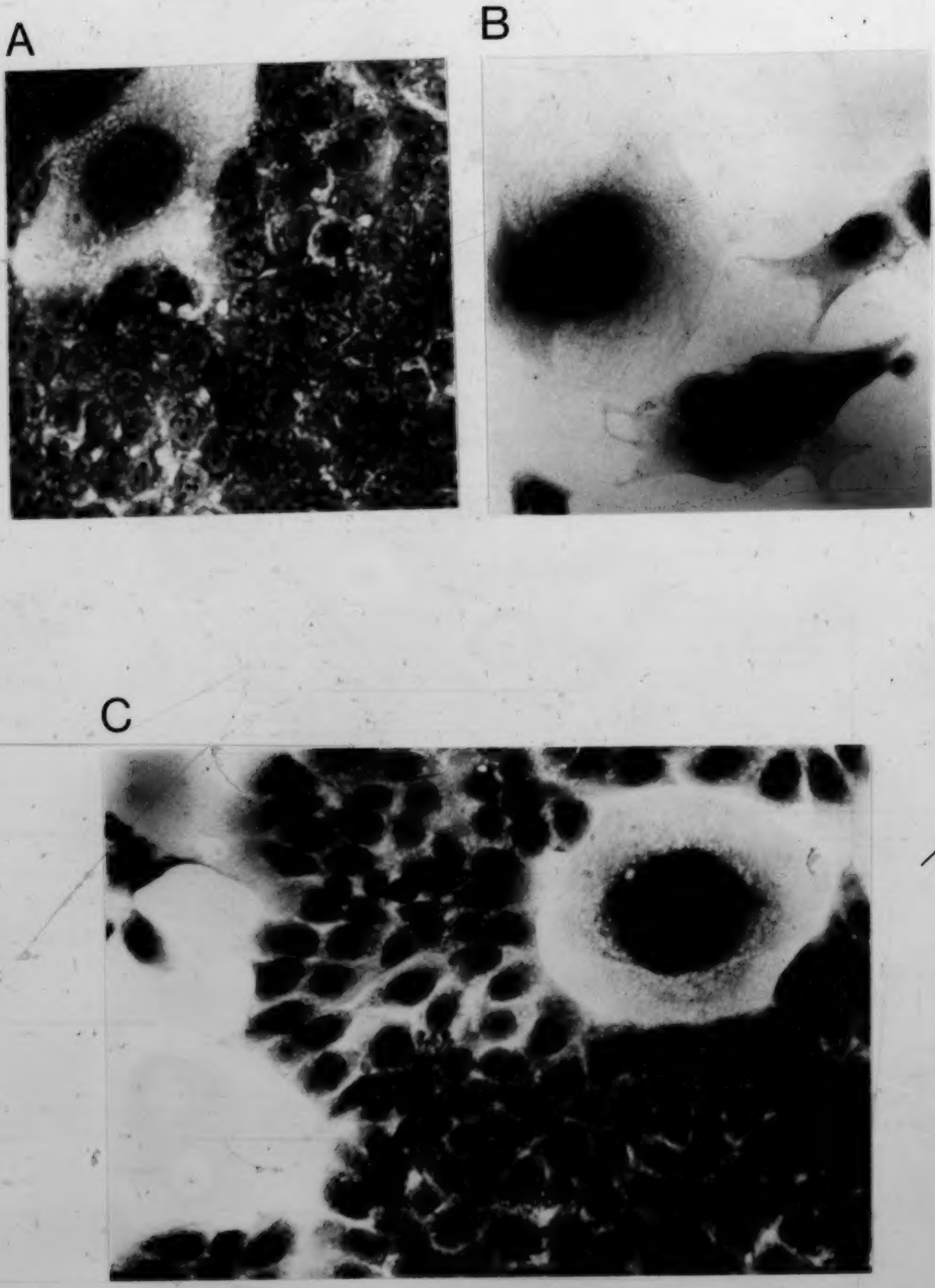
The mean (±S.D.) population doubling times of cultures exposed to methotrexate, hypoxanthine ($> 10^{-4}$ M) and thymidine (0.1 mM) were greater (27.4±2.4, n=7) than those of additive-free cultures (20.3±3.2, n=12).


A
Hx + 0.1 mM dThd
Hx
80
60
40
20
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Hypoxanthine (M)
Percent Change in Cell Number
B
d Thd + 1.0 mM Hx
d Thd
80
60
40
20
0
-20
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Thymidine (M)

thymidylate synthesis in the BeWo cell line, and are consistent with the earlier demonstration that thymidylate synthesis and *de novo* purine synthesis were inhibited following exposure to methotrexate (sections B.1 and B.2, respectively).

To determine if inhibition of dihydrofolate reductase was also the underlying cause of methotrexate-induced cytodifferentiation, the ability of thymidine and hypoxanthine or leucovorin to protect against the cytodifferentiative effects of methotrexate were assessed (Plate 4, Table 25). It is evident from Plate 4 that morphological differentiation to the STL phenotype was blocked by coadministration of hypoxanthine and thymidine. BeWo cells exposed to methotrexate in the presence of these additives were moderately sized, mononucleated, and otherwise morphologically indistinguishable from untreated CTL cells. Data from a large series of experiments are presented in Table 25 where it is evident that hypoxanthine and thymidine or leucovorin blocked expression of cytodifferentiative markers and allowed proliferation of BeWo cells in the presence of methotrexate.

Plate 4. Effects of hypoxanthine and thymidine on methotrexate-induced differentiation to the STL phenotype. BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with additive-free growth medium (dFBS) or medium (dFBS) containing methotrexate with or without thymidine and hypoxanthine. After 48-hr exposures, cultures were stained with Wright's stain for microscopy as described in Materials and Methods. **A**: no additives. **B**: 1 μM methotrexate. **C**: 1 μM methotrexate plus 1 mM hypoxanthine and 0.1 mM thymidine. Final magnification was 250x.


A
B
C

To assess the relative importance of purine versus thymidylate starvation during methotrexate-induced BeWo cytodifferentiation, the ability of thymidine or hypoxanthine alone to prevent the effects of methotrexate was investigated (Table 25). Either purine or thymidylate starvation resulted in inhibition of BeWo cell proliferation, but only thymidylate starvation resulted in expression of cytodifferentiative markers.

The effects of fluorodeoxyuridine, a specific inhibitor of thymidylate synthetase, were examined to test the hypothesis that depletion of cellular thymidylate during methotrexate exposure was responsible for induction of the CTL-to-STL response (Table 26). Fluorodeoxyuridine has been previously shown to induce morphological differentiation of BeWo cells.[11] Exposure of BeWo cells to fluorodeoxyuridine inhibited proliferation and induced a cytodifferentiative response similar to that of methotrexate, including increases in cell volume, stimulated expression of heat-stable alkaline phosphatase, and morphological differentiation to the STL phenotype. Thymidine blocked, to varying degrees, all of these effects. These results suggest that thymidylate starvation was the primary biochemical effect of methotrexate.

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[11] Personal communication, Dr. S. J. Friedman.

Table 25

Capacity of Hx, dThd, and Leucovorin to Protect Against the Cytodifferentiative and Antiproliferative Effects of Methotrexate

	Methotrexate-free				Methotrexate-treated			
Additive	Number of Doublings	Modal Volume (pl/cell)	Heat-stable Alk. Phos.	Predominant Phenotype	Number of Doublings	Modal Volume (pl/cell)	Heat-stable Alk. Phos.	Predominant Phenotype
none	2.20 ± 0.41 (22)[1]	3.34 ± 0.72 (27)	17.1 ± 1.0	CTL	0.04 ± 0.18 (7)	6.49 ± 1.47 (13)	108.9 ± 0.5	STL
Hx	2.10 ± 0.58 (8)	3.38 ± 1.43 (12)	19.2 ± 0.6	CTL	0.34 ± 0.31 (16)	10.4 ± 1.43 (17)	110.0 ± 13.8	STL
dThd	2.20 ± 0.23 (4)	2.50 ± 0.11 (3)	9.7 ± 2.1	CTL	0.36 ± 0.01 (3)	2.82 ± 0.36 (3)	39.0 ± 2.0	CTL
Hx + dThd	1.75 ± 0.16 (4)	2.67 ± 0.30 (3)	12.6 ± 0.2	CTL	**1.76 ± 0.17 (7)**	**4.54 ± 1.78 (5)**	**37.1 ± 2.3**	**CTL**
Leucovorin	2.78 ± 0.01 (4)	3.90 ± 0.41 (3)	n.d.	CTL	**2.36 ± 0.25 (4)**	**3.45 ± 1.29 (3)**	**n.d.**	**CTL**

[1]*number of separate determinations.*

BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, cell numbers were determined with an electrical particle counter and culture fluids were replaced with drug-free growth medium (dFBS) or medium (dFBS) supplemented with methotrexate (1 μM), hypoxanthine (0.1 or 10 mM, Hx), thymidine (0.10 mM, dThd), or leucovorin (0.1 mM). (Materials and Methods, section A.1). After 48-hr exposures, cell numbers and volumes (average±S.D., section B.1), the specific activity (nmol *p*-nitrophenyl phosphate released/min/mg protein) of heat-stable alkaline phosphatase (average±S.D.; n=3 cultures assayed in triplicate, section G) and the predominant cell phenotype (n=3 cultures; 100 cells/culture, section A.1) were determined as described in Materials and Methods. The number of population doublings (mean±S.D.) was calculated from cell numbers determined at the beginning and end of exposures.

Table 26

Capacity of Thymidine to Protect Against the Cytodifferentiative and Antiproliferative Effects of Fluorodeoxyuridine

Additive	Number of Doublings	Modal Volume (pl/cell)	Heat-stable Alk. Phos.	Predominant Phenotype
none	3.68 ± 0.03 (3)[1]	3.00 ± 0.02	23.6 ± 0.8	CTL
dThd	2.80 ± 0.06 (3)	2.95 ± 0.13	n.d.	CTL
Fudr	0.19 ± 0.23 (6)	7.03 ± 0.50	85.6 ± 5.1	STL
Fudr + dThd	**3.43 ± 0.02 (3)**	**4.05 ± 0.28**	**43.5 ± 1.1**	**CTL**

[1] *number of separate determinations*

BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with drug-free growth medium (dFBS) or medium (dFBS) containing 4 μM fluorodeoxyuridine (Fudr) and/or 0.1 mM thymidine (dThd) and cell numbers were determined (Materials and Methods, section A.1). After 72-hr exposures, cell numbers and volumes (average±S.D., n=3, section B.1), the specific activity (nmol *p*-nitrophenyl phosphate released/min/mg protein) of heat-stable alkaline phosphatase (average±S.D.; n=3 cultures assayed in triplicate, section G), and the predominate cell phenotype (n=3 cultures; 100 cells/culture, section A.1) were determined as described in Materials and Methods. The number of population doublings (mean±S.D.) was calculated from cell numbers determined at the beginning and end of exposures.

C. Summary

Although the BeWo cell line was derived from a choriocarcinoma obtained from a patient that developed resistance to methotrexate chemotherapy, cultured BeWo cells were sensitive to the primary biochemical effects of the drug. Incorporation and protection experiments and direct measurement of DNA, RNA, and protein (see Chapter V for determination of RNA and protein) content indicated that exposure to methotrexate inhibited *de novo* thymidylate, purine, and folate-dependent amino acid synthesis of BeWo cells. These effects, which are normally considered cytotoxic, did not lead to cell death (see Chapter V for viability studies) but, instead morphological and biochemical markers associated with the syncytiotropoblast were expressed by methotrexate-treated BeWo cells.

Quantitative cell-cycle analysis demonstrates that inhibition of proliferation by methotrexate was a result of an S-phase block in the BeWo cell cycle. During short-term exposures to methotrexate (< 8 hr), an increase in the relative number of cells in G_1 indicated that initiation of DNA synthesis was inhibited. However, this block was overcome with continued exposure to drug. A transitory inhibition of initiation of DNA synthesis has been previously observed with cytosine arabinoside inhibition of Chinese hamster ovary cell proliferation (29). The molecular mechanism by which cells overcome inhibition of DNA synthesis is not known (29).

It has been suggested that transcription or translation of chorionic gonadotropin or placental alkaline phosphatase might occur in one discrete portion of the cell cycle (30). Quantitative analysis of the effects of methotrexate on progression of BeWo cells through the cell cycle provided evidence that cell cycle effects could account for increases in expression of a specific protein. After 24 or 48 hr-exposures to 1 μM methotrexate, the number of cells present in S_1 phase of the cell cycle increased about 2.5-fold. During 48-hr exposures to 1 μM methotrexate, it was demonstrated (Chapters VI-IX) that methotrexate stimulated a 2.6 to 5-fold increase in alkaline phosphatase activity. Thus, if early S-phase cells expressed greater levels of heat-stable alkaline phosphatase, increased expression of the enzyme could be accounted for by an increase in the number of cells in a particular phase of the cell cycle.

The biochemical effects of methotrexate exposure on one-carbon metabolism of BeWo cells indicated that dihydrofolate reductase was sensitive to inhibition by methotrexate; hence, protection experiments were undertaken to determine if depletion of cellular thymidylate and purines was responsible for induction of the CTL-to-STL cytodifferentiative response. Leucovorin or hypoxanthine and thymidine restored proliferative capacity and blocked expression of markers of the CTL-to-STL cytodifferentiative response. Although the protective effects of leucovorin may be more complex than merely replenishment of tetrahydrofolate pools (see Chapter 2), protection against the cytodifferentiative effects by both thymidine and hypoxanthine or a source of reduced folate indicates that disruption of folate-dependent one-carbon metabolism is necessary for methotrexate-induced BeWo cell differentiation.

To assess the relative importance of thymidylate versus purine starvation during induction of the CTL-to-STL differentiative response, the effects of hypoxanthine or thymidine alone on methotrexate's antiproliferative and cytodifferentiative effects were assessed. Results of these experiments indicated that either purine or thymidine starvation resulted in inhibition of proliferation, but thymidylate starvation was the biochemical effect of methotrexate responsible for induction of the CTL-to-STL response. The hypoxanthine requirements of BeWo cells during methotrexate exposures for cell growth during the differentiative response were similar to those required for continued proliferation since, in the presence of excess thymidine, the concentration of hypoxathine (ca. 50 μM) required for proliferation was similar to the concentration of hypoxanthine (80 μM) required for half-maximal restoration of the ability of dialyzed serum to support increases in cell volume.

Hypoxanthine and thymidine or leucovorin, but not hypoxanthine alone, have been previously shown to block methotrexate-stimulated expression of syncytiotrophoblastic markers by cells derived from choriocarcinoma (30, 31) Administration of thymidine (the growth medium contained hypoxanthine) or leucovorin blocked stimulation of both human chorionic gonadotropin secretion and placental alkaline phosphatase activity in methotrexate-treated BeWo cells (30). Similarly, thymidine (the growth medium contained hypoxanthine) blocked methotrexate-stimulation of chorionic gonadotropin secretion in JAr cells, another cell line derived from human choriocarcinoma (31).

To test the hypothesis that thymidylate starvation was responsible for methotrexate-induction of the CTL-to-STL differentiative response, the effects of flurodeoxyuridine, a potent inhibitor of thymidylate synthetase, on proliferation and expression of cytodifferentiative markers were investigated. Fluorodeoxyuridine was previously shown to induce formation of giant cells and morphological differentiation of BeWo cells.[12] In this work, it was demonstrated that fluorodeoxyuridine, at a concentration that inhibited proliferation, also caused differentiation to the STL phenotype, which was accompanied with increases in cell volume and expression of heat-stable alkaline phosphatase. These differentiative effects were blocked by administration of thymidine.

In conclusion, methotrexate apparently induced a thymineless and purineless state in BeWo cells, which did not lead directly to cell death. Instead, characteristics associated with the *in utero* syncytiotrophoblast were expressed. Since the protective nature of the nonproliferative state toward drugs that kill cycling cells is a well established phenonenon (32-34), it was possible that the differentiative response of BeWo cells alters their sensitivity to the cytotoxic effects of methotrexate. Resistance to methotrexate cytotoxicity by induction of the STL phenotype which is nonproliferative, may represent a novel biological mechanism of resistance to methotrexate.

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[12] Personal communication, Dr. S. J. Friedman.

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VIII. Effects of Population Density on the BeWo Cytodifferentative Response

A. Introduction

During previous investigations (Chapter V and VI), it became apparent that culture conditions altered the response of BeWo cells to methotrexate. Most notably, dialysis of serum used to supplement the basal growth medium and increases in the number of cells per culture reduced expression of syncytiotrophoblastic markers during exposure to methotrexate. The population density of cultures alters cell motility, shape, proliferation, and differentiation (1-6). Density-dependent inhibition of proliferation is the most studied of population density effects, and explanations for this phenomenon include depletion of growth factors (7), accumulation of toxic factors or chalones (8), decreased availability of nutrients from culture fluids (9-12), and changes in cell-cell or cell-substratum interactions (13-14). Following exposure to methotrexate, the surface of BeWo cells is changed and cell-substratum interactions are altered, suggesting that culture microenvironment may alter formation of giant cells during the CTL-to-STL differentiative response (15).

In this work, experiments were undertaken to determine the basis of the inhibition of the CTL-to-STL response observed in crowded BeWo cultures. Methotrexate-induced increases in cell volume were inversely related to population size. Since it was previously shown that exogenous hypoxanthine was required for formation of giant cells (Chapter VI), culture fluids were supplemented with 1 mM hypoxanthine. Hypoxanthine augmented methotrexate-induced increases in BeWo cell volume, but this effect was diminished at high population densities. Although there appeared to be sufficient drug available in crowded cultures to affect a response, drug uptake was less at higher population densities. Since a number of parameters varied in crowded cultures, a methodology was developed to manipulate population densities within cultures of constant cell number, volume of growth medium, and culture substratum. Using this culture system, methotrexate-induced increases in cell volume and expression of alkaline phosphatase were shown to be inversely related to population density. The viabilities of BeWo cells in sparsely and densely populated cultures were the same, indicating that decreased expression of differentiative markers was not due to cell death. The effects of population density on utilization of adenine and hypoxanthine indicated that salvage of purines from culture fluids was less in crowded cultures, but it was not possible to determine if this was a cause or an effect of the density-dependent inhibition of the CTL-to-STL differentiative response.

B. Results

1. Giant cell formation in crowded cultures

The effects of culture population on methotrexate-induced giant cell formation were first assessed by simply increasing the number of cells in the inoculum used to establish BeWo cultures (Method A; Materials and Methods, section A.3). When BeWo cultures of different populations (2×10^5 - 1×10^6 cells/T-25 flask) were exposed to 1 μM methotrexate, the relative increase in cell volume was inversely related to the number of cells per flask (Figure 25). After 48-hr exposures, the volume of methotrexate-treated BeWo cells ranged from 10 pl/cell at the lowest density (2×10^5 cells/flask) to 4 pl/cell at the highest density (8×10^5 cells/flask). Since methotrexate-induced formation of giant cells was previously shown to require salvage of preformed purines (Chapter VI), hypoxanthine was added to the growth medium to determine if reduced formation of giant cells in densely populated cultures was related to depletion of purines. Hypoxanthine augmented methotrexate-induced increases in cell volume, but this effect was diminished at higher population densities.

Thus, relatively small increases in the number of cells per culture appeared to inhibit methotrexate-induced expression of cytodifferentiative markers by BeWo cells. To minimize this effect, all previously reported experiments in this work used cultures that were established at an initial population density of $< 1.33 \times 10^4$ cells/cm^2.

Since population density, cell number, and the ratio of cell number to volume of growth medium all varied in cultures established with Method A, the results presented in Figure 25 do not unequivocally demonstrate that formation of giant cells was inhibited in a density-dependent manner. It was possible that increased cell numbers resulted in depletion of unidentified serum factors or methotrexate from culture fluids. Because the affinity of methotrexate for dihydrofolate reducatase is sufficiently high that the enzyme titrates available drug (Chapter II), the amount of "free" drug may have been insufficient in crowded cultures

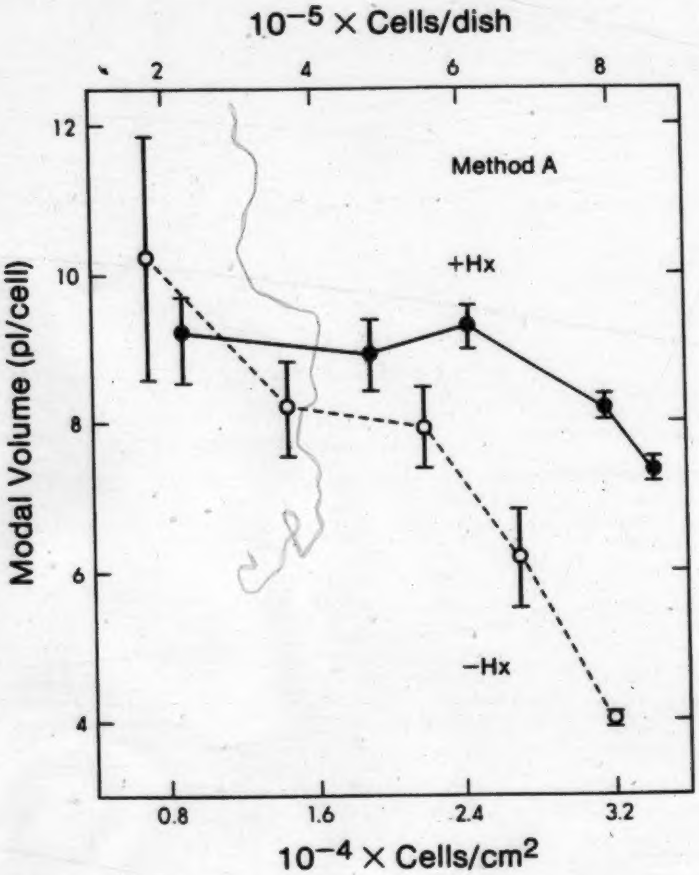

Figure 25. Effects of culture population and hypoxanthine (Hx) on methotrexate-induced increases in cell volume. BeWo cultures were established with 2 - 10 x 10^5 cells/T-25 flask (Method A; Materials and Methods, section A.3), and after 24 hr, culture fluids were replaced with growth medium (5 ml) containing 1 μM methotrexate (○) or medium (5 ml) containing 1 μM methotrexate and 1 mM hypoxanthine (●). After 48-hr exposures, modal cell volumes (average ± S.E., n=3) were determined electrically (Materials and Methods, section B.1). Population densities were calculated from the number of cells present in cultures after 48-hr drug exposures.

to elicit a response.

To determine if sufficient amounts of methotrexate were available to saturate cellular targets of the drug, the concentration of methotrexate remaining in culture fluids after 48-hr exposures was determined by high performance liquid chromatography. The chromatographic system used afforded excellent separation of methotrexate from UV-absorbing material present in growth medium (Figure 26). The results presented in Table 27 illustrate that methotrexate was depleted from culture fluids during 48-hr exposures at all population densities, and uptake of methotrexate from culture fluids was decreased at high cell densities. While the amount of "cell-associated" methotrexate was reduced by about 30% at higher densities, previous experiments (Chapter VI, section B.5) suggested that, at the lowest population density, there was sufficient methotrexate in culture fluids to induce formation of giant cells. Exposure of low density cultures to one-tenth the concentration of methotrexate used in Table 27 resulted in near-maximal increases in cell volume.

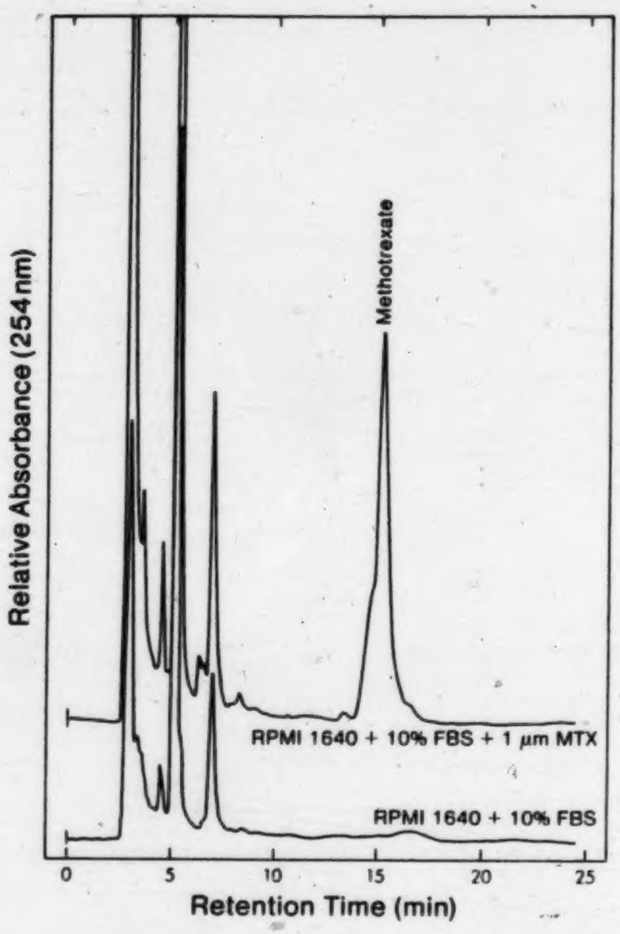

Figure 26. Chromatograpic separation of methotrexate from UV-absorbing material present in growth medium. Samples of growth medium with or without 1 μM methotrexate were fractionated with an ion-exchange chromatography procedure described in Materials and Methods (section F.2). Methotrexate-containing effluents were concentrated, and methotrexate was separated from other UV-absorbing material by isocratic elution of a Waters μBondapak C_{18} column with acetate (0.2 M, pH 5.5) buffer:methanol:acetonitrile (85.3:3.4:6.3) at a flow rate of 1.5 ml/min (Materials and Methods, section F.2).

Table 27

Effects of Culture Population on the Amounts of Methotrexate Remaining in Culture Fluids After 48-hr Exposures

Cell Inoculum ($\times 10^{-5}$)	Cells per Culture ($\times 10^{-5}$)	Methotrexate (μM)	"Cell-associated" Methotrexate (fmol/cell)
2	1.76 ± 0.04	0.86 ± 0.16	6.4
4	3.63 ± 0.41	0.68 ± 0.13	4.4
6	5.51 ± 0.80	0.81 ± 0.05	1.7
8	6.77 ± 0.61	0.72 ± 0.07	2.1
10	7.96 ± 0.34	0.68 ± 0.06	2.0

BeWo cultures were established and exposed to methotrexate as described in Figure 25. After 48-hr exposures, cell numbers were determined, and culture fluids were collected for analysis of methotrexate by high performance liquid chromatography as described in Figure 26. Values presented are mean (±S.D.) number of cells per flask (n=3) and methotrexate concentration (n=3). When drug-exposures were initiated, the concentration of methotrexate in growth medium was 1.0±0.2 μM (n=6), as determined chromatographically. "Cell-associated" methotrexate was calculated from amounts of drug depleted from culture fluids and number of cells per culture at 48 hr. Insignificant binding of methotrexate to the culture flasks occured during 48-hr exposures (data not shown).

2. Effects of population density on expression of cytodifferentiative markers and cell viability

To eliminate the possiblity that depletion of unidentified serum factors or methotrexate resulted in the apparent density-dependent inhibition of giant cell formation, an experimental approach was developed that allowed manipulation of population density as the only experimental variable (Method B; Materials and Methods, section A.3) Cultures established with Method B were compact dome-like structures consisting of several multilayers of cells. The average density of "spot cultures" established with Method B was reproducible for a given inoculum volume. For example, the coefficient of variation for the area of 8 "spot cultures" established with 2.0 x 10^5 cells in a 0.1 ml inoculum was less than 11%. Dishes that showed evidence of cells spreading from the central dense "spot culture" were discarded.

It is apparent from the experiment presented in Figure 27 that giant cell formation was inhibited in densely populated cultures with constant cell numbers. At the lowest density, the modal volume of methotrexate-treated cells was greater than 10 pl/cell, whereas at higher densities ($>$ 7.0 x 10^4 cells/cm^2), modal volumes of drug-treated cells were the same ($p>0.05$) as that of proliferating BeWo cells (3.51±1.29 pl/cell, n=20). In these experiments, the volume of growth medium, concentration and absolute amounts of methotrexate, number of cells, and available culture substratum were virtually identical for all cultures, indicating that methotrexate-induced formation of giant cells was inhibited in a density-dependent manner.

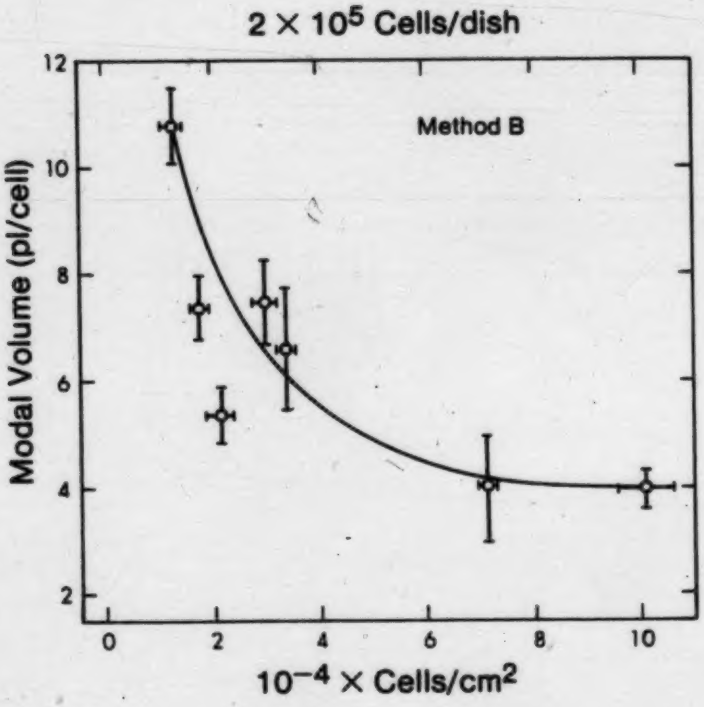

Figure 27. Effects of population density on methotrexate-induced increases in BeWo cell volume. BeWo cultures were established with 2 x 10^5 cells/60 mm tissue culture dish with 0.05 to 5 ml inocula (Method B, Materials and Methods, section A.3), and after cells had attached, additional growth medium was added to a total of 5 ml/culture. After 24 hr, culture fluids were replaced with growth medium (5 ml) containing 1 μM methotrexate, and after 48-hr exposures, modal cell volumes (average±S.D., n=3) were determined electrically (Materials and Methods, section B.1). Population densities (mean±S.D, n=3) were calculated from the area of the culture substratum occupied by cells and the number of cells remaining after 48-hr exposures to methotrexate.

The effects of population density on expression of heat-stable alkaline phosphatase, another marker of the BeWo cytodifferentiative response, were also examined in cultures established with Method B (Table 28). At sparse population densities (ca. 1 x 10^4 cells/cm^2), continous exposure to 1 μM methotrexate resulted in a 7-fold increase in the specific activity of heat-stable alkaline phosphatase in cell extracts, whereas at higher densities (ca. 7 x 10^4 cells/cm^2), the activity only increased 2.3-fold over control values. The apparent activity of heat-stable alkaline phosphatase in drug-free cultures was unaffected by population density ($p>0.05$).

Cell death occurs in the core of tumors (16, 17) and cultured tumor-cell spheroids (18), probably because of decreased availability of nutrients. Thus, it was possible that cell death in the core of the "spot cultures" was responsible for decreased expression of BeWo cytodifferentiative markers. Cell viability was assessed by vital-staining methods in sparsely and densely populated cultures established with Method B (Table 29). Since the viability of BeWo cells in drug-free or methotrexate-treated cultures was not affected by increased population density, reduced expression of markers of the BeWo cytodifferentiative response was not due to cell death and necrosis in the core of dense cultures.

Table 28

Effects of Population Density on Methotrexate-induced Expression of Heat-stable Alkaline Phosphatase

Culture	Specific Activity Untreated	 MTX-treated
Sparse	21.2 ± 4.3	146.3 ± 30.4
Dense	32.5 ± 14.3	76.3 ± 32.6

BeWo cultures were established with 2 x 10^5 cells/60 mm tissue culture dish with 5 ml (sparse) and 0.1 ml inocula (dense), and after cells had attached, an additional 4.9 ml of growth medium was added to dense cultures (Method B), as described in Figure 27. After 24 hr, culture fluids were replaced with drug-free medium (Untreated) or medium containing 1 μM methotrexate (MTX-treated). After 48-hr exposures, extracts of the cells were made, and the specific activity (nmol *p*-nitrophenyl phosphate released/min/mg protein) of heat-stable alkaline phosphatase (mean±S.D., n=9) was determined as described in Materials and Methods (section G). The population density (mean±S.D.) of sparse cultures was 1.2±0.2 x 10^4 cells/cm^2 (n=3) and of dense cultures was 6.5±0.7 x 10^4 cells/cm^2 (n=8).

Table 29

Effects of Population Density and 1 μM Methotrexate on Viability of BeWo cells

	Untreated		MTX-treated	
Vital Stain	Sparse	Dense	Sparse	Dense
Trypan blue	93 ± 4	97 ± 1	95 ± 3	90 ± 3
Fluorescein diacetate	98 ± 2	97 ± 2	92 ± 7	91 ± 3

BeWo cultures were established with 2 x 10^5 cells/60 mm tissue culture dish with 5 ml (sparse) and 0.1 ml inocula (dense), and after cells had attached, an additional 4.9 ml of growth medium was added to dense cultures, as described in Table 28. After 24 hr, culture fluids were replaced with drug-free medium (Untreated) or medium containing 1 μM methotrexate (MTX-treated). After 48-hr exposures, cultures were disaggregated by trypsinization, and cell viabilities (mean±S.D., n=3, 500 cells/culture) were determined by vital staining methods as described in Materials and Methods (section A.4).

3. Rate of purine salvage in sparsely and densely populated cultures

Since it was previously demonstrated that methotrexate-induced increases in cell volume were dependent on exogenous hypoxanthine, the rates of incorporation of adenine and hypoxanthine into RNA were measured to determine the relative ability of BeWo cells in sparsely and densely populated cultures to salvage purines. In the experiment of Table 30, cultures were established by Method B. Incorporation of hypoxanthine (50 μM) and adenine (5 μM) into RNA were inhibited, respectively, by approximately 60 and 80%. Increasing the extracellular concentration of hypoxanthine (1 mM) or adenine (1 mM) overcame the apparent inhibition of purine salvage. The experiments of Figure 27 and Table 29 were conducted in growth medium with an extracellular concentration of hypoxanthine (from fetal bovine serum used to supplement the basal growth medium) of 0.5 μM (see Chapter VI, section B).

Decreased cell surface in direct contact with the growth medium is thought to result in less efficient uptake of compounds present in the growth medium (9-12). Since initial rates of purine uptake could not be measured,[13] it was not possible to determine if decreased salvage of purines was a cause or an effect of density-dependent inhibition of the CTL-to-STL cytodifferentiative response. However, the experiments presented in Table 30 indicated that salvage-dependent RNA synthesis, previously shown to be necessary for formation of giant cells (Chapter VI), was less in densely, than in sparsely, populated cultures.

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[13] Zero-trans influx of purines into most cultured cells reaches equilibration within seconds (19), and it was not feasible to determine initial rates of purine uptake into BeWo cells over this time scale.

Table 30

Effects of Population Density on Salvage of Purine Bases

	cpm/10^5 cells		pmol/µg RNA	
Purine	Sparse	Dense	Sparse	Dense
Ade (5 µM)	7848 ± 305	1336 ± 48	3.00 ± 0.10	0.75 ± 0.12
Hx (50 µM)	6574 ± 559	2679 ± 75	6.53 ± 0.50	3.93 ± 0.59
Ade (1 mM)	1090 ± 160	700 ± 35	62.1 ± 3.1	57.6 ± 9.0
Hx (1 mM)	304 ± 9	193 ± 19	58.5 ± 1.7	53.8 ± 9.4

BeWo cultures were established with 2 x 10^5 cells/60 mm tissue with 5 ml (sparse) and 0.1 ml inocula (dense), and after cells had attached, an additional 4.9 ml of growth medium was added to dense cultures, as described in Table 28. After 24 hr, culture fluids were replaced with drug-free medium (Untreated) or medium containing 1 µM methotrexate (MTX-treated). After 48-hr exposures, culture fluids were replaced with medium containing [8-^{3}H]adenine (5 µM; 144,000 cpm/nmol, 1 mM; 990 cpm/nmol) or [8-^{3}H]hypoxanthine (50 µM; 56,000 cpm/nmol, 1 mM; 290 cpm/nmol), and after 30 min, intervals of incorporation were ended by aspiration of culture fluids, rinsing with 0.15 M NaCl, and acid-precipitation with cold 0.2 M perchloric acid (Materials and Methods, section C.3). Values represent the mean (±S.D.) of cpm incorporated per 10^5 cells or pmol incorporated per µg RNA. The number of cells and RNA content, respectively, were for sparse cultures, 2.13±0.06 x 10^5 cells/culture (n=6) and 38.3 µg RNA/culture (n=2), and for dense cultures 2.10±0.03 x 10^5 cells/culture (n=6) and 26.4 µg RNA/culture (n=2).

C. Summary

In the work presented in this Chapter, the effects of culture population on methotrexate-induced expression of cytodifferentiative markers were shown to be a result of both exhaustion of exogenous hypoxathine and density-dependent effects unrelated to global culture population or the ratio of cell number to volume of culture fluids. Although spontaneous differentiation to the STL phenotype is relatively rare in drug-free BeWo cultures, it too appears to be inhibited at higher population densities (20). At low densities, the number of giant cells and multinucleated cells were 4 and 2.5%, respectively, of the total population, whereas at higher densities, the number of giant cells and multinucleated cells were 1.5 and <0.2% of the total population (20). Thus, methotrexate-induced and spontaneous differentiation to the STL phenotype are apparently sensitive to culture microenvironment. Cytotrophoblastic cells are surrounded by syncytiotrophoblast in the villi of normal placenta and choriocarcinoma is characterized by central cores of cytotrophoblast-like cells with peripherial rims of syncytiotrophoblast-like cells (21) suggesting that tissue microenvironment may be an important determinant of cellular differentiation. Thus, density-dependent inhibition of BeWo cytodifferentiation may reflect control mechanisms of *in vivo* differentiation.

The original observation that methotrexate-induced expression of cytodifferentiative markers was less in crowded cultures was apparently a result of both depletion of serum factors (hypoxanthine) and population density effects. As cell numbers per culture were increased (Method A), the cellular demand for purines apparently surpassed the amounts of hypoxanthine salvageable from serum present in growth medium. Above a threshold population density of approximately 2.5 x 10^4 cells/cm^2, formation of giant cells in cultures established with Method A was inhibited in a manner that could not be reversed, even by high concentrations (1 mM) of exogenous hypoxanthine. In cultures in which only local population density was varied (Method B), methotrexate-induced increases in cell volume and expression of heat-stable alkaline phosphatase were inversely related to density. The apparent decreases in uptake of methotrexate and salvage of exogenous adenine or hypoxanthine by methotrexate-treated BeWo cells indicated that utilization of molecules from culture fluids was less in crowded cultures. However, it was not determined if decreased utilization of purines or uptake of the inducer were effects or causes of density-dependent inhibition of the BeWo cytodifferentiative response.

BeWo cell membrane glycosylation patterns (22), cell agglutination with lectins (23), and adhesion to the culture substratum (15, 20) change during methotrexate exposure. In addition, susceptibility of the nuclear-cytoskeletal framework to extraction by potassium iodide, an actin-depolymerizing agent (24) and the organization of keratin filaments and actin stress fibers (15) also change during drug exposure. All of these properties would be affected by changes in cell-cell or cell-substrate interactions. Although the mechanism of density-dependent inhibition of the BeWo CTL-to-STL differentiative response is unknown, it has been demonstrated that culture microenvironment altered the response of BeWo cells to methotrexate.

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IX. Conclusions

During exposure to methotrexate, BeWo cells undergo a cytodifferentiative response that is analogous to development of *in utero* quiescent syncytiotrophoblast. The current investigation is concerned with relationships between methotrexate toxicity and differentiation in the BeWo cell line. Results of this investigation indicate that BeWo cells were exceedingly sensitive to the antiproliferative effects of methotrexate ($IC_{50}=50$ nM), but relatively resistant to its cytotoxic effects ($IC_{50}>10$ μM). Drug exposures that completely inhibited proliferation also inhibited thymidylate synthetase, *de novo* purine synthesis, and progression through S-phase of the cell cycle. Protection experiments indicated that disruption of folate-dependent one-carbon metabolism resulted in thymidylate and purine starvation and concommitant inhibition of proliferation. Although thymidylate or purine starvation usually kill cells within 4-48 hr, viability of methotrexate-treated BeWo cells was unaffected after 48-hr exposures to methotrexate at concentrations supramaximal for inhibition of proliferation. Following removal of drug; cells reverted to the CTL phenotype and regained proliferative capacity. Resistance to methotrexate cytotoxicity by induction of the STL phenotype, which is nonproliferative, may represent a novel biological mechanism of resistance to methotrexate.

Experiments undertaken to identify the biochemical effect(s) of methotrexate responsible for induction of the BeWo cytodifferentiative response indicated that thymidylate starvation stimulated expression of syncytiotrophoblastic markers. Depletion of purines present in the culture fluids antagonized methotrexate-induced increases in cell volume, but did not alter expression of heat-stable alkaline phosphatase or morphological differentiation.

During this investigation it became evident that expression of syncytiotrophoblastic markers was also antagonized by increases in cell population during exposure to methotrexate. This phenomenon was a result of both depletion of hypoxanthine from culture fluids and density-dependent effects unrelated to absolute culture populaton. Utilization of methotrexate and purines from the culture fluids suggested that decreased availability of molecules may be important in density-dependent inhibition of cytodifferentiation, but the large number of effects of methotrexate on the BeWo cell surface and cell-substratum interactions suggests that culture microenvironment may have more profound effects on the CTL-to-STL cytodifferentiative response.

This work demonstrates that methotrexate induced non-toxic cytodifferentiative changes in BeWo cells through a mechanism that is toxic to most cultured cells. These results provide a foundation for further studies on the cytodifferentiative mechanism of action of methotrexate in the BeWo cell line. It is not intuitively obvious how thymidylate starvation altered regulation of expression of syncytiotrophoblastic markers, but the effects of the drug were unrelated to toxicity indicating that the response was not an "agonal gasp". One possibility is that methotrexate disrupted methonine and adenylate metabolism altering synthesis of S-adenosyl methionine and methylation patterns. Since methylation reactions are important for cell regulation, disruption of its metabolism could induce a pleiotropic biological response. Another possibility, related to a area of intensive research, is that methotrexate exposure altered expression of cellular oncogenes. It is rapidly becoming evident that certain oncogenes are intimately related to embryonic genes that may control the "proliferative-differentiative" status of the cell, and decreased expression of cellular oncogenes correlates with acquisition of differentiated function and loss of malignant potential in certain culture human tumor cells (1-4). Although the molecular mechanisms of control of proliferation and differentiation are poorly understood, research in this area could have profound consequences on the therapy of cancer.

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Appendix 1

Fidelity of the Coulter Method for Determination of BeWo Cell Volume

A. Introduction

Resistance pulse sizing, invented by Coulter in 1953, is a convenient method for determining cell volume (1). In this study, the Coulter method was used to quantitate giant cell formation, a marker of the CTL-to-STL differentiative response. Since values for BeWo cell volume have not been previously reported, conditions for accurate determination of the volume of these cells were established. To measure volume electrically, the shape of the particle must be known; however disaggregated BeWo cells were irregularly shaped. Since diameters can only be optically determined in two-dimensions, the shape of BeWo cells could not be defined. To determine the effects of variations in particle shape on calculation of Coulter volumes, the mathematical relationship between particle shape, volume, and magnitude of the resistance pulse was described. From theoretical calculations, it became apparent that small deviations in axial ratio would cause relatively small errors in Coulter determination of particle volume. Values of BeWo cell volume obtained electrically were similar to values obtained with two independent methods for determining cell volume, indicating that the Coulter method yielded accurate measures of BeWo cell volume.

B. Theory of Resistance Pulse Sizing

The physical basis of resistance pulse sizing is that particles suspended in an electrolyte contribute to the net resistivity of the compound medium (2). In practice, particles suspended in an electrolyte are pumped through a small diameter orifice. An electrode is placed on either side of the orifice, and a constant current is maintained with an alternating current power supply. As each particle passes through the electrical field, electrolyte is displaced thereby changing the resistance across the orifice (3). By Ohm's law, altered resistivity is detected as a change in voltage across the two electrodes. If the specific resistivity of the particle is greater than that of the electrolyte, increased resistance as the particle passes through the orifice is accompanied by a transitory increase in voltage.

Simplified resistivity relationships predict that small particles passing through a Coulter orifice cause changes in voltage directly proportional to their volume (4). In practice, these relationships were inadequate since differently shaped particles of the same volume yielded different changes in voltage in a model system of the Coulter orifice (5). More precise analysis of resistivity relationships indicate that particle resistivity, shape, and orientation to the electrical field can affect the amplitude of the voltage pulse (1, 5-9). Maxwell derived an equation describing the conductivity of a compound medium consisting of spherical particles suspended in an electrolyte (2). Solving this equation for the special case of a single particle yields an equation that describes the relationship between the change in resistance (or amplitude of the voltage pulse) and the volume of a spherical particle as it migrates through a Coulter orifice.

Maxwell's equation is:

$$r_1 = \frac{2r_2 + r_3 + \;\; P(r_2 - r_3)}{2r_2 + r_3 - 2P(r_2 - r_3)} \cdot r_3 \qquad [1]$$

Where r_1, r_2, and r_3 are the specific resistivities of the suspension, particle, and electrolyte, respectively, and P is the volume fraction of the suspended particles.

Solving for P:

$$P = \frac{(r_1 - r_3)(2r_2 + r_3)}{(r_3 + 2r_1)(r_2 - r_3)} \qquad [2]$$

To introduce the change in resistance:

$$R_1 = R_3 + \Delta R_2 \qquad [3]$$

Where R_1 and R_3 are the resistance of the orifice filled with the suspension and electrolyte, and ΔR_2 is the change in resistance caused by migration of the particle through the orifice.

For an orifice with cross-sectional area, A, and length, L:

$$r_1(L/A) = r_3(L/A) + \Delta R_2 \qquad [4]$$

Thus:

$$r_1 = r_3 + \Delta R_2(A/L) \qquad [5]$$

Equation 5 is introduced into equation 2 (1). Upon rearranging:

$$P = \frac{\Delta R_2\,(2r_2 + r_3)}{2\Delta R_2\,(r_2 - r_3) + 3r_3\,(L/A)(r_2 - r_3)} \qquad [6]$$

Assuming $R_3 >> \Delta R_2$, and $P = \Delta V/LA$ where ΔV = volume of the particle:

$$\Delta V = (1/r_3)(\Delta R_2 \cdot A^2)[r_3/3r_2 - 2r_2/3r_3 + 1/3] \qquad [7]$$

ΔR_2 is measured as $\Delta U/i$, where ΔU is the absolute magnitude of the voltage pulse and i is the aperture current.

Substituting $\Delta U/i$ for ΔR_2 and rearranging equation 7:

$$\Delta V = \frac{\Delta U \cdot A^2}{r_3 \cdot i} \cdot \frac{1}{\frac{3[1 - r_3/r_2]}{[2 + r_3/r_2]}} \qquad [8]$$

To introduce the form factor *(f)*:

$$f = \frac{3[1 - r_3/r_2]}{[2 + r_3/r_2]} \qquad [9]$$

For a nonconducting particle, $r_2 >> r_3$ and equation 9 reduces to:

$$f = 3/2 \qquad [10]$$

Thus, the final equation for a nonconducting spherical particle is:

$$\Delta V = \frac{\Delta U \cdot A^2}{r_3 \cdot i} \cdot \frac{1}{3/2} \qquad [11]$$

For spheroids with three different axis, an an equation analgous to equation 1 is (8):

$$r_1 = \frac{r_2 + (f' - 1)r_3 + (f' - 1)(r_2 - r_3)P}{r_2 + (f' - 1)r_3 - (r_2 - r_3)P} \cdot r_3 \qquad [12]$$

which reduces to:

$$\Delta V = \frac{\Delta U \cdot A^2}{r_3 \cdot i} \cdot \frac{1}{f'} \qquad [13]$$

Equations 11 and 13 describe the mathematical relationship between the magnitude of the voltage pulse (ΔU) and the volume (ΔV) of nonconducting spheres and spheroids. It is evident that the voltage pulse resulting from migration of a particle through the orifice is dependent on the shape and resistivity of the particle, the resistivity of the electrolyte, the cross-sectional area of the orifice, the aperture current, and the orientation of the particle in the orifice. In practice, absolute voltages are not measured. Instead, the Coulter counter is calibrated with particles of known volume and shape, and the volume of an unknown particle is determined relative to that of the calibration particle. With the same voltage amplification, aperture current, electrolyte, and orifice, the volume of the unknown particle ($\Delta V''$) is:

$$f'' \cdot \Delta V'' = f \cdot \Delta V \cdot (\Delta U''/\Delta U) \qquad [14]$$

Where f'' and $\Delta U''$ are the form factor and voltage pulse of the unknown particle, and f, ΔU, and ΔV are the form factor, voltage pulse, and volume of the calibration particle.

It is evident that volume cannot be determined from the magnitude of the electrical resistance voltage pulse without assuming particle shape. For nonconducting spheres, the form factor is 3/2, and for spheroids the form factor is a constant related to the ratio of the axes of the particle. Simplified equations (6) for the form factor of regular ellipsoidal particles oriented perpendicular to the electrical field are:

for an oblate spheroid (principal axes a, b, b, $m = a/b < 1$)

$$1/f' = \frac{m \cos^{-1} m}{(1 - m^2)^{1.5}} - \frac{m^2}{(1 - m^2)} \qquad [15]$$

for a prolate spheroid (principal axes a, b, b, $m = a/b > 1$):

$$1/f' = \frac{m^2}{(m^2 - 1)} - \frac{m (\cos^{-1} m)}{(m^2 - 1)^{1.5}} \qquad [16]$$

Since the form factor (f') is a constant based on the ratio of the three axes of the particle and not their absolute lengths, relative sizes can be determined if the particles have the same shape.

Form factors for prolate spheroids with differing ellipticity and orientations to the electrical field are presented in Figure 28. If a particle of constant volume is transformed into an increasingly elongated prolate spheroid, it is evident that equation 11 results in an overestimation of true particle volume if the long axis is oriented perpendicular to the electrical field. Conversely, if the long axis is parallel to the electrical field, the volume of a prolate spheroid is underestimated by using a shape factor of 3/2. If particles are randomly oriented these two effects cancel, and the calculated volume closely approximates the true volume of the particle.

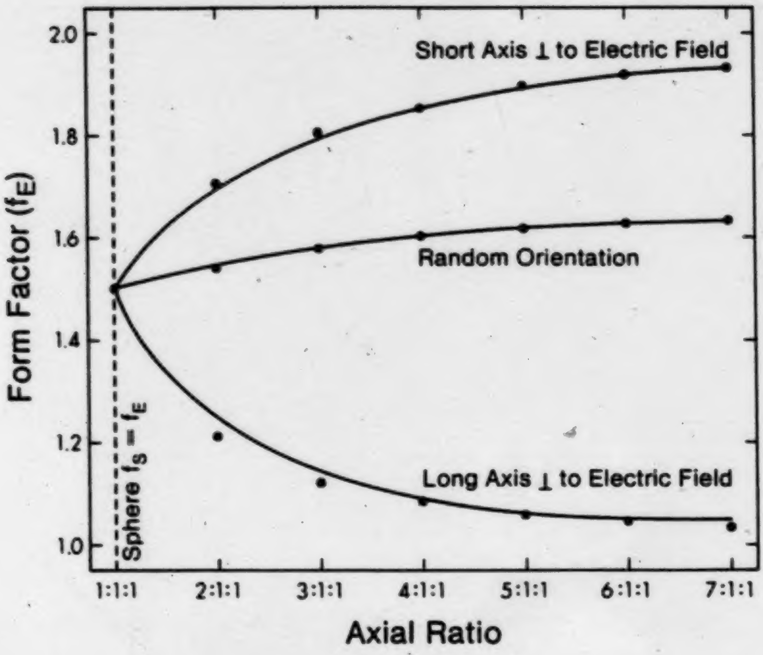


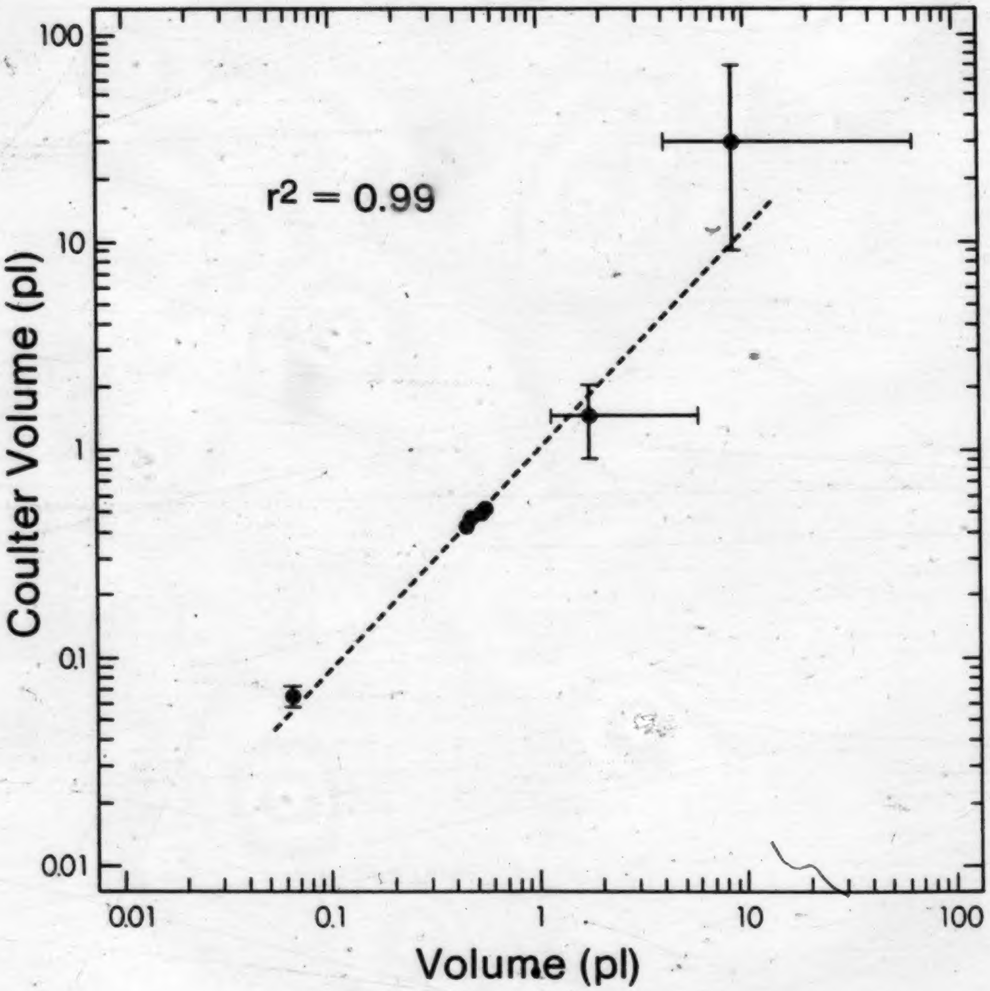
Figure 28. Form factors of prolate spheroids of different ellipticity. The hydrodynamic flow through a Coulter orifice is perpendicular to the electrical field; thus, the orientation of the particles under sizing conditions varies between random and long axis perpendicular to the field depending on the degreee of orientation caused by hydrodynamic forces.

C. Results

1. Relationship between resistance change and volume of microspheres

The magnitude of the resistance voltage pulse is linearly related to particle volume only when particles are small in comparison to the diameter of the Coulter orifice. When the ratio of particle to orifice diameter is greater than 0.3, distortion of the electric field (the basis of the voltage pulse) caused by a particle as it migrates through the orifice is influenced by the orifice wall (10). The relationship between particle volume and the amplitude of the voltage pulse for the Coulter counter used during this investigation was determined using microspheres of known diameters (Figure 29). There was a linear relationship between the Coulter volume of microspheres and the volume of the particle calculated from its diameter over a range of 0.065 to 8.9 pl/particle (particle:orifice diameter ratio of 0.05 to 0.257). Virtually all of the volume measurements obtained for BeWo cells fall within this range.

Figure 29. Relationship between Coulter volume and volume of microspheres calculated from their reported diameters. Microspheres of diameters ranging from 5 to 25.7 μm were suspended in 0.15 M NaCl by repeated pipetting and sonication and were electronically sized with a Coulter counter fitted with a 100-μm (diameter) aperture tube. Plotted is the relationship between mean Coulter volume ± S.D. (vertical error bars) obtained from the Coulter volume distribution and the calculated volume of microspheres (horizontal error bars are the ranges of volume calculated for particles with diameters ± 1 S.D. from the mean diameter of the particle). Points without error bars indicates that the S.D. was smaller than the size of the symbol. The dashed line and the coefficient of determination (r^2) were determined by linear regression using the method of least squares.


r^2 = 0.99
Coulter Volume (pl)
100
10
1
0.1
0.01
0.01
0.1
1
10
100
Volume (pl)

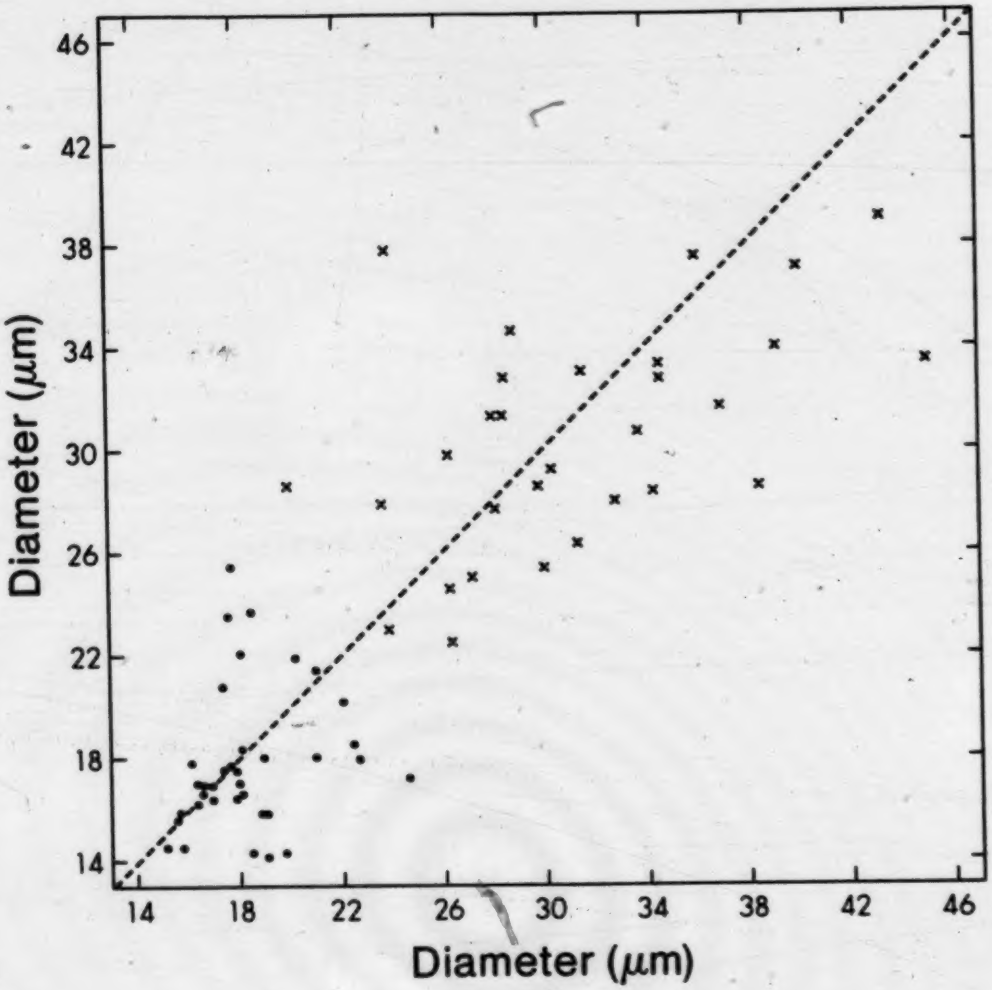
2. Conditions for determination of BeWo cell volume

It is apparent from equations that describe the relationship between particle volume and magnitude of the resistance pulse that *a priori* knowledge of particle shape is necessary to calculate cell volume with the Coulter method. The major and minor diameters of BeWo cells were measured from photomicrographs to determine the shape of disaggregated cells (Figure 30) Both proliferating and methotrexate-treated BeWo cells were irregularly shaped following trypsinization and suspension in 0.15 M NaCl. However, the average axial ratios of untreated and drug-treated BeWo cells were the same; thus, if particles were not orientated during the wet-mounting process, then proliferating and drug-treated BeWo cells possess the same shape. Since it was previously shown (eq. 14) that the form factor cancels out of the volume equation if particles have the same shape, it is evident that *relative* changes in BeWo cell volume can be accurately determined with the Coulter method.

To estimate the errror introduced into Coulter volume measurements by small variations in axial ratio, the apparent volumes of randomly-oriented prolate and oblate spheroids were calculated as if the particles were spherical. Using equation 11 (which assumes that the unknown particle fits the ideal case of a nonconducting spherical particle (form factor=3/2), the Coulter volume of a prolate ellipsoid with axial ratio 2:1:1 would be overestimated by 3% and that of an oblate ellipsoid with axial ratio 2:2:1 would be overestimated by 6%. If particles are assumed to be oriented in the orifice, the error increases depending on orientation in the electrical field. Thus, small deviations in axial ratio from that of the ideal case, such as were observed with BeWo cells (axial ratio $<$ 1.3:1.3:1), would be expected to cause small errors in calculation of Coulter volume. Consequently, BeWo cell volumes were calculated using a form factor of 3/2, corresponding to the ideal case of a nonconducting sphere, and were reported on an absolute scale in this investigation.

Experimental conditions for precise determination of the volume of BeWo cells were established before the effects of methotrexate on cell volume were measured. Accurate counting and sizing of osmotically fragile particles with the Coulter method requires stability of

Figure 30. Axial ratios of BeWo cells cultured in the presence and absence of methotrexate. BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with drug-free growth medium (○) or medium medium containing 1 μM methotrexate (●). After 24 hr exposures, cultures were disaggregated with trypsin-EDTA and suspended in 0.15 M NaCl. Following suspension, cells were mixed in a 1:1 numerical ratio with microspheres of known diameter (9.54 μm) suspended in 0.15 M NaCl, and the resulting mixtures were wet-mounted on microscope slides. Photomicrographs were taken of fields containing cells with at least one polystyrene microsphere, and the diameters of spheres and BeWo cells were measured on the photomicrographs with a vernier micrometer. The total magnification was 52-fold, and the coefficient of variation for the diameter of microspheres determined optically was 4% (n=30). The average axial ratios of proliferating cells (1.13±0.13, n=36) and methotrexate-treated cells (1.15±0.13, n=30) were the same (not significantly different, $p>0.05$). The dashed line represents a 1:1 relationship between the two diameters (perfect sphere).


46
42
38
34
30
26
22
18
14
Diameter (μm)
14
18
22
26
30
34
38
42
46
Diameter (μm)

the particles and isotonicity with the suspending electrolyte. The effects of suspension in 0.15 M NaCl on BeWo cell volume are presented in Table 31 where it is evident that the volumes of control and methotrexate-treated BeWo cells were unchanged after 1 hr in saline. Furthermore, the volumes obtained in saline were the same as the volumes obtained when similarly treated cells were suspended in growth medium. All determinations of cell volume presented in this investigation were completed within 30 min of suspension in saline.

Accurate electrical determination of particle size over a large range of volumes is dependent on inherent linearity between aperture current and voltage amplification. Changes in BeWo cell volume during exposure to methotrexate were sufficiently large to require several aperture current or voltage amplification settings. Thus, it was necessary to establish the range of linear response of the voltage amplifier. It is evident from results presented in Table 32 that mean volumes determined for BeWo cells were virtually identical over a 16-fold range of aperture current. The volumes of control BeWo cells were routinely determined with an aperture current of 0.125 milliamperes, whereas the volumes of methotrexate-treated cells were determined with currents of 0.016 to 0.125 milliamperes, depending on their size.

Table 31

Effects of 0.15 M NaCl on BeWo Cell Volume

Time (min)	Cell Volume (pl/cell)	
	Untreated	MTX-treated
5	3.59 ± 1.17 (3)[1]	11.08 ± 4.97 (3)
15	3.17 ± 1.36[2] (3)	n.d.
30	3.13 ± 1.50[2] (3)	10.82 ± 5.64[3] (1)
60	3.00 ± 1.46[2] (3)	10.42 ± 5.11[3] (1)
120	3.50 ± 1.31[2] (3)	9.79 ± 5.61[3] (1)

[1] *number of determinations.*

[2] *not significantly different (p $<$ 0.05) from control volume at 5 min.*

[3] *not significantly different (p $<$ 0.05) from drug-treated volume at 5 min.*

BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with drug-free growth medium (Untreated) or medium containing 1 μM methotrexate (MTX-treated). After 48-hr exposures, cultures were disaggregated with trypsin-EDTA. Disaggregated cells were suspended in 0.15 M NaCl, and mean cell volumes (average±S.D., n=number of separate determinations indicated) were determined electrically at the times indicated (Materials and Methods, section B.1). In a separate experiment (not shown), the mean cell volume (average±S.D., n=3) of BeWo cells suspended in medium supplemented with 10% fetal bovine serum (2.18±0.26 pl/cell) was the same (p>0.05) as that of cells suspended in 0.15 M NaCl (2.30±0.11 pl/cell).

Table 32

Effects of Aperture Current on BeWo Cell Volume

Aperture Current (mA)[1]	Mean Cell Volume (pl/cell)
0.016	3.59 ± 1.26
0.032	3.57 ± 1.24
0.063	3.56 ± 1.20
0.125	3.63 ± 1.21
0.250	3.59 ± 1.22

[1] *mA=milliamperes.*

BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, culture fluids were replaced with drug-free growth medium. After 48 hr, cultures were disaggregated by trypsinization, pooled, and cells were suspended in 0.15 M NaCl. Mean cell volumes (±S.D.) were determined at the aperture current indicated, as described in Materials and Methods (section B.1).

3. Optical and radiochemical determinations of cell volume

The accuracy of the Coulter method for measuring BeWo cell volume was assessed by comparing electrically determined values with values obtained using two independent methods. Since the diameter of BeWo cells could be optically measured in only two dimensions, an assumption regarding cell shape was necessary before volume could be calculated from diameter. The axial ratios of BeWo cells were irregular (Figure 30), hence the shape of the cells could not be precisely defined. However, if shape changes or orientation of cells did not occur during the preparation of wet-mounted slides for light microscopy, then upper and lower bounds for BeWo cell volume could be estimated. Assuming that the unknown third axis was equal to the major two-dimensional axis yielded an upper bound for BeWo cell volume, whereas assuming that the third axis was equal to the minor two-dimensional axis yielded a lower limit for BeWo cell volume. The range of cell volumes obtained with these calculations are summarized in Table 33.

It is evident from the considerations above that critical evaluation of the fidelity of the Coulter method for determining the volume of BeWo cells required a method that was independent of particle shape. Cell-associated water space represents the "osmotically active" volume of the cell. If hydrophobic compartments of the cell that exclude water (eg. lipid bilayer, condensed chromatin, the interior of protein molecules) are similar in different cell types, then cell-associated water volumes and Coulter volumes should be linearly related for a number of different cells types. Since the cell-associated water volume does not represent the total volume of the cell, the ratio of this volume to Coulter volume should be less than 1. It is evident from the data presented in Figure 31, that cell-associated water volume and Coulter size were linearly related for all but one[14] of the cell types examined. The ratios of cell-associated

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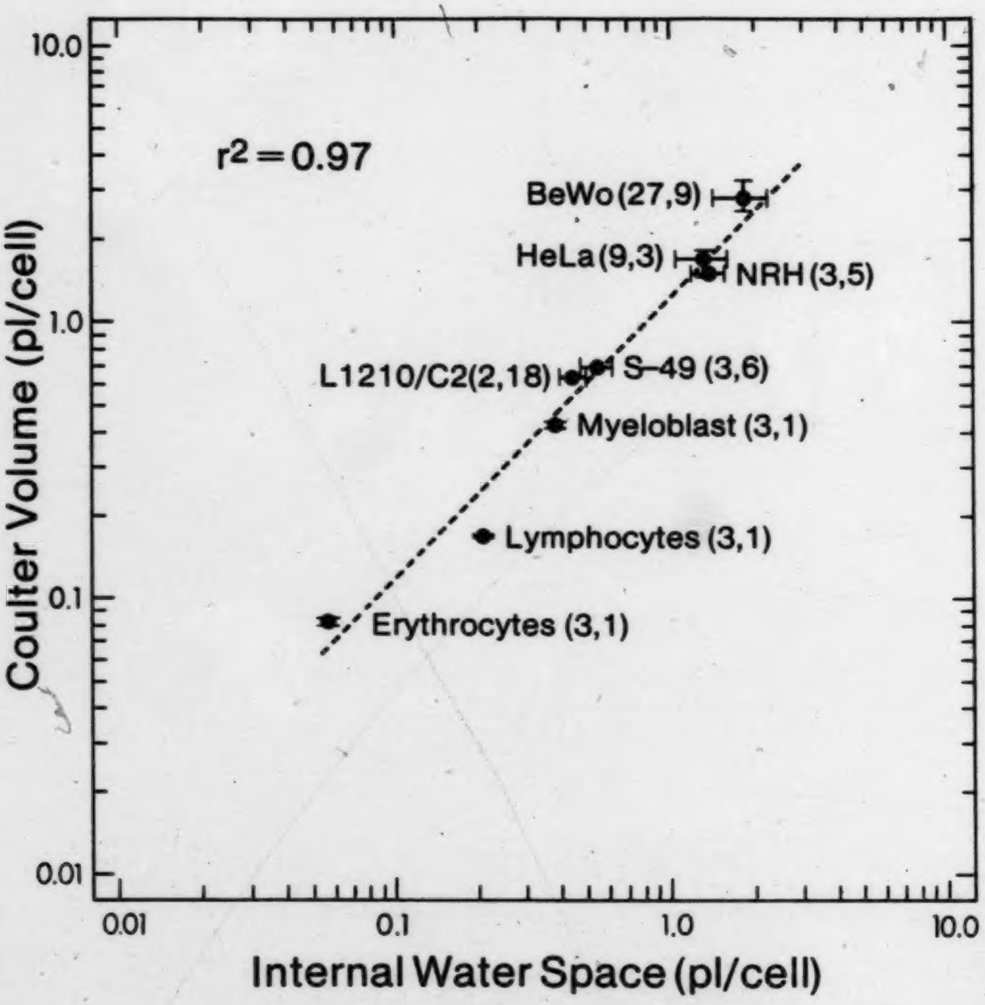
[14] The lymphocyte preparation examined had a cell-associated water:Coulter volume ratio of > 1. Contaminating cells in the preparation probably resulted in an overestimation of the cell-associated water volume of lymphocytes. Volume measurements of lymphocytes were excluded from all subsequent calculations of the ratio of Coulter to cell-associated water volume.

Figure 31. Relationship between Coulter volume and cell-associated water (Internal Water Space). Cultured cells were propagated and collected for cell volume measurements and blood cells were isolated as described in Materials and Methods (section A). Coulter and cell-associated water volumes were determined as described in Materials and Methods (section B). Each symbol represents the average (±S.D.) cell volume for the number of determinations indicated (number of separate Coulter mean volume determinations, number of triplicate determinations of cell-associated water volume). Vertical error bars indicate the interassay S.D. in determination of mean Coulter volume, and horizontal error bars indicate the interassay S.D. in determination of mean cell-associated water volume. Points without error bars indicates that the S.D. was less than the size of the symbol. The dashed line and the correlation coefficient (r^2) were determined by linear regression using the least squares method.


r^2=0.97
BeWo(27,9)
HeLa(9,3)
NRH (3,5)
L1210/C2(2,18)
S-49 (3,6)
Myeloblast (3,1)
Lymphocytes (3,1)
Erythrocytes (3,1)
10.0
1.0
0.1
0.01
Coulter Volume (pl/cell)
0.01
0.1
1.0
10.0
Internal Water Space (pl/cell)

water volume to Coulter volume for cultured cells in monolayers (BeWo or Hela cells) or in suspension (L1210/C2, NRH, S49) and for human blood cells (erythrocytes or leukemic myeloblasts) were similar (mean ± S.D. = 0.79 ± 0.11, the range was 0.66-0.93, n = 7).

The immediate objective of these experiments was to assess the accuracy of the Coulter method for determining cell size. It is evident from the results summarized in Table 33 that the values of cell volume for both control and methotrexate-treated cells determined electrically, optically, and radiochemically were similar. While no single method afforded significant theoretical advantages, the Coulter method was the most practical.

The differences between Coulter and cell-associated water volume[15] were assumed to represent the hydrophobic partial volume of cells and were used to calculate an "intracellular volume" for BeWo cells. The value (average ± S.D.) for "intracellular volume" (2.40 ± 0.58, n = 9 triplicate determinations) or mean Coulter volume (2.83 ± 1.30, n = 27) of BeWo cells were the same in parallel experiments ($p > 0.05$).

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[15] The average hydrophobic compartment of the cells examined in these experiments (excluding lymphocytes) was 20.9% ± 10.3% of their Coulter volume, n = 7 cell types. To calculate the "intracellular volume" of BeWo cells, the average hydrophobic compartment of cells was added to the mean cell volume determined in a parallel experiment.

Table 33

Determination of BeWo Cell Volume

Method	Volume (pl/cell) Untreated	Volume (pl/cell) MTX-treated
Mean Coulter	2.83 ± 1.30 (27)[1]	10.28 ± 1.27 (9)[1]
Optical (sphere)	3.16	15.0
Optical (prolate spheroid)	2.97	14.4
Optical (oblate spheroid)	3.34	16.4
Cell-associated water	1.86 ± 0.40 (9)[2]	n.d.[3]

[1] *average (± S.D.) of the number of separate determinations of mean Coulter volume indicated.*

[2] *average (± S.D.) of the number of triplicate determinations.*

[3] *not determined.*

BeWo cultures were established with 2 x 10^5 cells/T-25 flask, and after 24 hr, cultures fluids were replaced with drug-free growth medium (Untreated) or medium containing 1 μM methotrexate (MTX-treated). After 48-hr exposures, cultures were disaggregated by trypsinization and cell volumes were determined electrically, optically, and radiochemically as described in Materials and Methods (section B.). Cell volumes were calculated from the diameters measured for Figure 30 by assuming that the unknown third axis was the same as either the major two-dimensional axis (oblate spheroid with axes of a,b,b where $a/b>1$) or the minor two-dimensional axis (prolate spheroid with axes of a,b,b where $a/b<1$) or by averaging the major and minor two-dimensional axes and assuming that cells were spheres.

D. Summary

Although the potential of electrical resistance pulse sizing has not been realized in its most imaginative setting, i. e., it was envisioned that the number and size of fish in a salmon run could be determined (4), the Coulter method is used to determine the volume of many biological and industrial particles. Among the biological particles, the volumes of erythrocytes, leukocytes, platelets, bacteria, spermatozoa, vaginal cells, virus, mitochondria, and tissue culture cells have been determined (1,4). In this work, the fidelity of the Coulter method for determination of the volume of BeWo cells was assessed by comparing Coulter volumes with cell volumes determined optically and radiochemically. Although the Coulter method is dependent on particle shape, calculations had suggested that moderate variations in axial ratio (up to 2:1:1 or 2:2:1) cause relatively small errors in determination of volume from the magnitude of a particle's voltage pulse. Similar values for the volumes of methotrexate-treated and untreated BeWo cells were obtained by three independent methods, indicating that the Coulter method could be used to accurately measure the volume of BeWo cells on an absolute scale under the conditions employed in this study.

The apparent discrepancy between Coulter and intracellular water volume is because the later is not a measure of total cell volume. The water-excluded, or osmotically-inactive volume, reported in the literature (11) is similar to the difference between Coulter and cell-associated water volume reported in this chapter. The osmotically inactive fraction of cells can be determined by measuring the volume of cells in hypertonic media (12), and the average (±S.D.) value for 7 different neoplastic and normal cells was 31.9 ± 6.5% of the total cell volume (11). Thus, the difference between Coulter and cell-associated water volume could be attributed to the partial volume of cells that excludes water.

In conclusion, the Coulter method has been established as a valid measure of the volume of BeWo cells. In work presented in subsequent Chapters, this method was used to quantitate formation of giant cells, a marker of the CTL-to-STL differentiative response of BeWo cells.

E. References

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Appendix 2

Relative Cellular Fluoresence Gated on Light Scatter

A. Introduction

In previous work (Chapter VII, section B.3), the effects of methotrexate on the relative DNA content of BeWo cells was assessed. It is evident from the distributions presented that a considerable number of "particles" present in methotrexate-treated cultures had DNA contents greater that 4N. Although microscopic examination of cell suspensions indicated that aggregates were present (ca. 10-20%), STL cells are apparently polyploid (1) and may contribute to fluoresence in the 4N, and greater, region of distributions. Hence, the light scatter characteristics and DNA content of particles present in BeWo cell suspensions were assessed to determine if light scatter could discriminate between single cells containing 4N and greater DNA contents (ca. 3-4% of the total population) and aggregates consisting of cells with 2N to 4N DNA contents (Figures 32 and 33)

Forward-angle light scatter is related to cell volume and can be used to characterize the DNA content of particles of a specific size (2). A single distribution that was slightly skewed to the right was obtained when the forward-angle light scatter of particles present in suspensions of BeWo cells was measured. Moving the lower gate[16] to successively larger channel numbers resulted in distributions with relatively more fluoresence in the 4N, and greater, region (data not shown). However, since there were no distinct subpopulations in these distributions, gating on forward-angle light scatter did not discriminate between single cells and aggregates. Right-angle (90°) light scatter is related to the internal architecture of cells (2). Although the 90° light scatter of particles present in BeWo cell suspensions was

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[16] The gating procedure involved collecting the fluorescence of "particles" with light scatter greater than a given channel number.

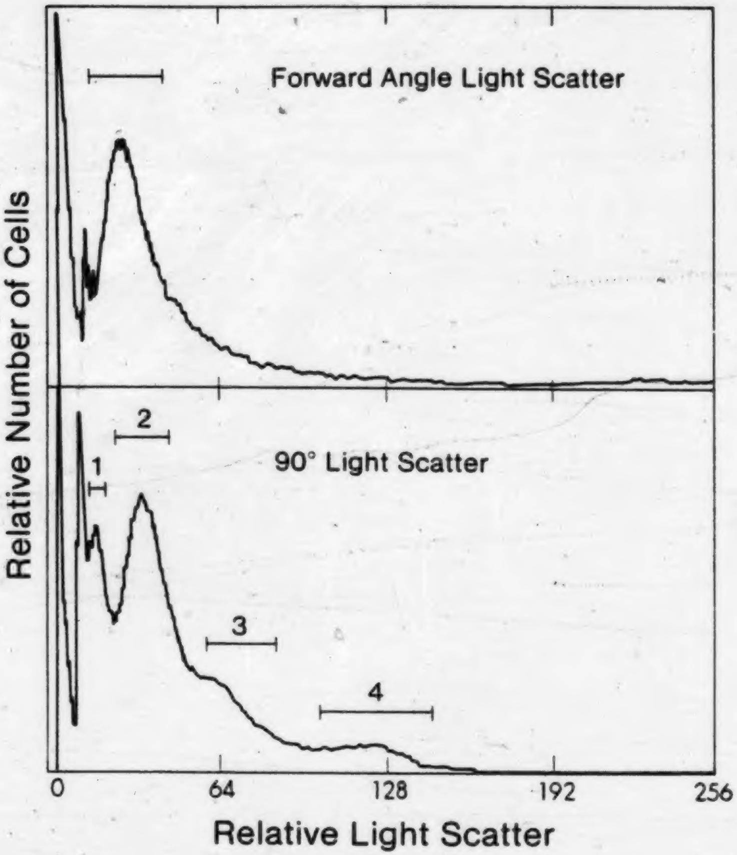

Figure 32. Light-scatter properties of particles present in suspensions of BeWo cells. BeWo cultures were established with 1 x 10^6 cells/T-75 flask and, after 72 hr, were disaggregated by trypsinization. Cells were pelleted, suspended in 0.15 M NaCl, fixed with ethanol, and stained with mithramycin as described in Figure 30. Distributions are representative of forward-angle and 90° light scatter of 457 nm wavelength incident light by BeWo cells or cell aggregates.

distributed as four distinct peaks (Figure 32) the DNA distributions of particles in these four regions were the same as those obtained without gating (data not shown). Thus, collecting fluorescence gated on 90° light scatter did not provide a method for identifying polyploid STL cells.

In an attempt to discriminate between a single 4N cell and a doublet of 2N cells, DNA distributions were collected by gating on both forward-angle and 90° light scatter (Figure 33). DNA distributions obtained with these gating procedures were the same as distributions obtained without gating (data not shown). Analysis of the data of Figure 33 indicated that each of the subpopulations had the same proportion of cells with a 4N content of DNA (Table 34). Thus, although 4 distinct subpopulations of BeWo cells were identified on the basis of their light-scatter characteristics, the DNA distribution (Figure 33) and 4N DNA content (Table 34) of each light-scatter subpopulaton was similar to that of the total population of cells.

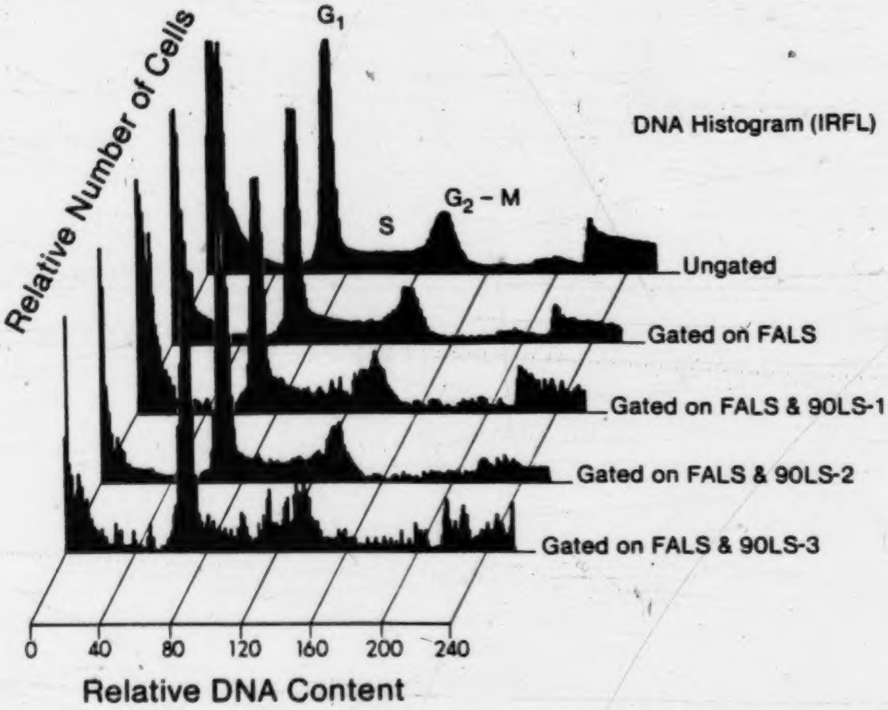

Figure 33. Relative DNA content of cells with specific light scatter characteristics. The fluorescence distributions of mithramycin-stained BeWo cells with the light-scatter characteristics indicated in Figure 32 were obtained with a EPICS V flow cytometer. To compare DNA distributions obtained by gating on forward-angle light scatter (FALS) and 90° light scatter (90LS), distributions were smoothed with the least squares spline algorithim of the PARA1 program; 2N DNA content peaks were translocated to channel 60, and the areas of the 2N to 4N region (channels 40 to 160) were normalized to a constant value. The 2N to 4N distribution represents 10,000 to 50,000 cells. The increased noise evident in the two distributions gated on FALS and 90LS-2 (peak 2) or 90LS-3 (peak 3) was a result of decreased cell numbers.

Table 34

Relative Number of Cells With a 4N DNA Content
Present in DNA Histograms Obtained by Gating on Light Scatter

Light Scatter Characteristics	Percentage
Ungated	22.5
FALS	20.5
FALS + 90°LS-1	19.5
FALS + 90°LS-2	19.7
FALS + 90°LS-3	21.7

The proportion of cells present in the 4N distribution of DNA distributions presented in Figure 32 was determined with the PARA2 program.

C. References

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